

NIH: a strategy for the 21st century?

When the US National Institutes of Health get a new leader, important decisions will have to be made about funding strategies and research support if they are to continue as a paradigm of scientific excellence.

CONTRARY to trends of recent decades, the research budget of the US National Institutes of Health (NIH), now \$10.3 billion, is not likely to keep up with inflation next year when it is expected to receive an increase of only 3.3 per cent. Furthermore, most of that meagre increase has already been allocated to special causes through a process well-known as political 'earmarking'. Often thought of as a tendency of the Congress (where earmarked projects are known as 'pork'), this time it is clear that the administration of President Bill Clinton is every bit as capable of playing politics with research budgets as is the Congress.

Clinton's budget proposal, which cumulatively would reduce funding for research in the basic biomedical sciences broadly while focusing dollars on AIDS, women's health (particularly breast cancer), minority health and the human genome project — all areas that are fashionable these days and supported by influential lobbying or advocacy groups, whereas other scientifically ripe disciplines (neuroscience, for example) are left behind. It would appear that the Clinton administration has accepted the notion that targeted research will buy cures for disease. (In other agencies, notably the National Science Foundation, Clinton has requested substantial increases.)

In this environment, NIH needs a strong leader whose experience is well-grounded in serious research and who has the personal and administrative talent it will take to guide the institutes through difficult times. To its credit, the administration has acquiesced in the idea of a search committee of academics to find a replacement for the current head of NIH, cardiologist Bernadine P. Healy, whose resignation was requested by the White House. Healy will leave at the end of June. Meanwhile, the search committee, named by Donna Shalala, the Secretary of Health and Human Services, and Philip R. Lee, who has been nominated but not confirmed as assistant secretary for health, has been diligently combing the country for Healy's successor.

The search committee, headed by biologist Bruce Alberts (who will become president of the US National Academy of Sciences in July) has the confidence of the biomedical research community, where morale is at an all-time low despite the excitement of this unusually productive era in science. And the people with whom the committee has talked about the NIH directorship have the credentials to be taken seriously as candidates. Among them are William

Danforth, chancellor of Washington University in St Louis; Harold Varmus of the University of California at San Francisco (who would be the first NIH director to hold a Nobel prize); Yale University provost Judith Rodin; Patricia K. Donahoe of Harvard's Massachusetts General Hospital; Stanford University dean David Korn; and Herbert Pardes, dean of medicine at Columbia University. Others will be interviewed during the next few days.

At this point, this journal does not favour one candidate over another. However, it is imperative that President Clinton (or perhaps Hilary Rodham Clinton if the task falls to her) should confine themselves to the search committee's list of candidates when making a final selection. The administration's track record for appointments throughout the new government has been marred by over-zealous attention to the requirement that every list of potential job-holders "look like America", as Clinton puts it. The US biomedical research enterprise is one of the country's most valuable intellectual assets and its leader should be selected for qualities that match the intellectual and academic requirements of the job. It will be a sorry day if, in the end, the White House ignores the work of its NIH search committee.

One of the new director's first challenges will be to come to grips with Healy's most tangible legacy — a "strategic" plan for NIH research into the twenty-first century that is about to be released in the form of a glossy 100-page report called *Investment for Humanity*. Nearly two years in the writing, the strategic plan grew out of Healy's quite sensible idea that a \$10 billion enterprise, like any large business, should periodically take stock of itself in an organized fashion. That the plan got off on the wrong foot almost from the start because it was regarded by the outside community as the province of a hand-chosen group of Healy supporters within NIH should not detract from the rightness of its goal.

Today the NIH consists of 24 separate institutes, centres and divisions, most operating with a congressionally assigned budget and each only loosely responsible to the NIH director. Healy, perhaps influenced by her previous experience in the Republican White House where she served in the Office of Science and Technology Policy, approached strategic planning with a business executive's confidence. Not surprisingly, that rattled more traditional scientists who cling to the idea that science cannot be planned. In truth, to some extent it can. That is, one can identify areas of



scientific opportunity where good ideas are plentiful and imaginative experiments can be proposed.

But the tendency is to equate medical or social need with scientific opportunity and, to some extent, that has emerged in the final version of the plan, which also says the "right" things about most of modern biology. Thus, the health needs of women and minorities are given several paragraphs. But molecular medicine, computational biology, gene therapy and vaccine development are also emphasized, along with the need to attend to the training of the next generation of researchers and the importance of maintaining the public's trust in science by ensuring an enterprise that is ethical and free of fraud. In short, the plan (as might be inevitable) covers a good deal of scientific territory.

But more than that, it makes a couple of important and entirely controversial statements about the relationship between NIH and the US economy that should become the subject of open and informed debate among the research community. Namely, the plan states that NIH is (and should be) a major force in the US economy (through the pharmaceutical and biotechnology industries, for example) and it takes the line that "NIH discoveries and their applications have a positive impact on one of the Nation's most intractable social and economic problems — containing and reducing health care costs". On the face of it, that is not only an arguable statement but a questionable goal for an enterprise grounded in basic research that may not lack the will but does lack the means to keep any promise to reduce health care costs. Science does not work that way. Unless research leads to simple, inexpensive and complete prevention or cure of disease (childhood immunization comes to mind) it cannot take credit for major cost savings. Similarly, it is simplistic to blame high-technology medicine for rising costs. To suggest that NIH plays a significant part in the health care cost crisis is to misunderstand the complex financial and social elements of that crisis. NIH should stick to its scientific knitting. □

Free telecommunications

Europe's plan for electronic services had better be adventurous. If it is not, it will fail.

In the now standard European style, the telecommunications ministers of Europe will this week have been considering the future of their industry in the 12 member states. The logic is straightforward. If the concept of a free market means that German cabbages can be freely sold throughout Europe, then why not German telephone calls or Minitel boxes like those that have given France access to database services of a kind that ordinary people use? That is the theory, which the member states have in principle accepted. European telecommunications are indeed to be liberalized. But there is a long way to go before the principle will be turned into reality, despite the European Commission's hope that everything can be settled less than five years from

now, in time for 1 January 1998.

The difficulties are not merely that most major communications businesses in Europe are still closely identified with their governments, but that there are technical questions still unresolved. Residual chauvinism is nevertheless certain to be a hindrance. In many countries — France, Germany and Belgium, for example — the general opinion is for liberalization, but at some point in the future more distant than 1998. But much has changed in a short time. Even previously obdurate governments have been driven to sell their PTTs (postal, telephone and telegraph companies) to private owners by the difficulty of raising from public funds the capital they need to keep abreast of quickly moving technology. The idea that there should be international competition from within Europe is catching on. So why not also face up to the idea of competition from outside, from AT&T of the United States for example? That is where most PTTs find their nerves on edge.

With good reason. Both in Britain and in Denmark, telecommunications services are already controlled by private companies convinced that they have more to gain from open international competition than from a strictly European canvas. British Telecom, the chief British operator, is even seeking the right to provide trunk telephone and other services in the United States — and may be allowed to do so if AT&T is given a reciprocal right to operate in Britain. The most powerful impetus for a European policy is the fear that, without one, wider competition will simply take root and be ineradicable. That explains why change is in the air.

The technical obstacles to change are not unalloyed difficulties, but also opportunities. They consist of mobile telephone systems operated through Earth satellites, the use of cable television networks for carrying broad-band signals into homes and other premises and the prospect that the old dream of integrating computer systems with telecommunications networks will yet become a reality. The threat to the investment in the old order from mobile telephones is already substantial in Europe, accounting for one out of every eight telephone calls. And while most European governments have so far followed the United States in requiring that PTTs should not be allowed to distribute television signals, it is an inefficient use of resources to require that the increasingly sophisticated telephone network should be operated in parallel with a cable network that could do the same jobs.

What all this implies is that technological advances in the past few years guarantee that change is unavoidable, and will probably be more rapid than anybody has been supposing. Already, for example, there is nothing to prevent, say, a South Korean telephone company from providing Europe with telephone services by means of satellites launched from China. Whatever may be the present arrangements between European governments and their PTTs, the monopolies of the past would in practice be broken. That is why it would be best for Europe if its governments would go the whole hog, settling for radical competition and quickly. □

Britain weighs plan to axe US science mission

Washington. Plans by the Foreign Office to close the science and technology section of the British Embassy in the United States are likely to be scaled down thanks to fierce lobbying by UK science minister William Waldegrave and industry minister Michael Heseltine.

News of the plans, which arose from a routine government inspection earlier this

why is it science that has to be chopped?" said one official, who also expressed fears that the other three major diplomatic outposts of British science — in Paris, Bonn and Tokyo — could also be at risk.

Waldegrave and Heseltine, who both want to be seen as strong defenders of UK science and technology, have jointly pursued the issue in London and are hoping for a compromise whereby the section continues to function on a smaller scale. Supporters are concerned, however, that the compromise will leave the section so weak that closure will be only a matter of time.

Nick Browne, press attaché at the embassy, confirmed that the inspection had taken place and said that its results were still being evaluated. "There will be changes in the science section and elsewhere", he said, but declined to comment on the likely outcome. Peter Willis, a spokesman for the Foreign Office in London, said that "a final decision

has not yet been taken on the inspector's recommendations".

Neither spokesman would confirm details of either the inspectors' findings or any impending compromise. But a number of sources in Washington and London said that the inspectors had recommended closure.

The inspectors are senior former diplomats who conduct regular reviews of the entire UK diplomatic corps. A reversal of their judgement on political grounds would be highly unusual: in the words of one government official: "they are expected to get it right".

The science mission was originally engaged in the physical exchange of large quantities of scientific reports — up to 1,800 a month during the Second World War — but its staff was reduced in size as technology and alternative channels of exchange simplified this task. In recent years, however, the mission has become involved in arranging contacts between senior science officials in the two countries and monitoring US science policy developments.

Colin Macilwain

Congress agrees to new NIH office for AIDS programmes

Washington. A deal between the two houses of the US Congress has opened the way for an autonomous Office of AIDS Research (OAR) to administer the \$1 billion that the National Institutes of Health (NIH) spends annually on the disease. But it will not take effect unless Congress reconciles a dispute over whether those with HIV should be allowed into the United States — the only issue blocking passage of a bill to reauthorize NIH programmes.

The new AIDS office will be headed by a full-time director with authority to apportion NIH's AIDS budget among its institutes. At present, each is funded directly and the director of one of them — Anthony Fauci of the \$1 billion National Institute of Allergy and Infectious Diseases (NIAID) — is also the part-time director of the Office of AIDS Research, coordinating but not controlling spending.

The change has been sought by AIDS activists as a way to strengthen the government's efforts and is opposed by NIH officials, who see it as an unnecessary additional layer of bureaucracy. Fauci does not want the purely administrative job of OAR director, yet he cannot be pleased to lose control of the \$450 million in AIDS funding that goes to NIAID.

But one AIDS activist discounts rumours that Fauci is unhappy enough to leave NIH. "Tony wants to keep his laboratory at NIAID and talk of him quitting is a scare tactic", says Mark Harrington of the Treatment Action Group in New York. "I think when the president signs the bill, he'll go along with it." Fauci was unavailable for comment.

If the bill goes through, the NIH office will be one of two new elements in the fight against the disease. The other is an 'AIDS czar', yet to be named, who will work directly for President Bill Clinton in the White House.

AIDS activists are concerned that the dispute over the NIH bill may delay AIDS spending for the 1994 fiscal year that begins on 1 October. But a spokeswoman for NIAID said that those plans are ready to be implemented, that financial planning for 1995 is already under way and that the budget for fiscal year 1996 would be the first under the control of the new OAR director.

The proposal to establish an autonomous Office of AIDS Research is part of a complex NIH reauthorization bill, of which the sole remaining disagreement is a provision that would bar the immigration into the United States of those with HIV. Staff of both houses were meeting again this week to seek a compromise on that issue.

Colin Macilwain

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Sir Henry Tizard (right) and US Army Lt Gen. Eddy attend a post-war British civil-defence planning exercise.

year of British diplomatic service activity in the United States, emerged last week as the section celebrated its fiftieth birthday — making it the longest-established unit of its type anywhere in the world. The section was formally opened in 1943, shortly after Sir Henry Tizard arrived in Washington to exchange information on radar, fission and other military technology.

British ambassador Christopher Meyer did not mention the closure plan at a fiftieth birthday reception held at his residence, at which he praised the past and present work of the section. In the 1950s, the section had 11 diplomats and 20 locally hired staff, but it has since scaled back to three diplomats, led by science and technology counsellor Don Rolt, and a small support staff.

Waldegrave, whose Office of Science and Technology is currently producing a White Paper (policy document) on science policy, led the fight to save the section after senior officials in scientific government departments in London objected to the closure. "We all know that funding is tight, but

Britain's Wellcome Trust stretches its financial wings

London. University College, London (UCL) has accepted the offer of a £30 million (US\$47 million) loan from the Wellcome Trust to buy and convert the historic building that houses its original teaching hospital into an international centre for biomedical research (see next page).

The loan, believed to be the largest sum ever offered by a private foundation to a college of the University of London, is the latest in a series of moves by Wellcome that are intended to establish centres of excellence in biomedical research throughout Britain. Such generosity has been made possible by the sale last summer of £2.1 billion in shares of the pharmaceutical company Wellcome plc. The trust, now the richest medical research charity in the world with a portfolio valued at £5.4 billion, has also made a recent gift of £270 million to the Burroughs Wellcome Fund in the United States (see below).

The increased income means that the trust now has more than £200 million a year to spend on medical research — double the figure of a year ago. It supports fields ranging from parasitology to neurosciences but not cancer research, which is seen as receiving adequate funding from other charities. It has special programmes in areas considered to be underfunded, such as toxicology, mental health and tropical medicine.

In principle, trust officials say, there has been little change in the way it is spending its money, complementing government

funding for medical research by providing generous fellowships and equipment grants to university scientists. In practice, the magnitude of its income — making the trust's annual budget even larger than that of the Medical Research Council (MRC) — is allowing it to pursue policies designed to make a major impact on medical research in Britain.

For example, the trust is in the process of re-vamping its fellowship schemes to address the problems caused by the lack of teaching jobs in universities and the growing number of researchers working on short-term contracts. The idea is to create a complete 'parallel' career structure for university biomedical scientists.

A judicious mixture of carrot and stick is being used to persuade universities to back the trust's approach. Some of its grants, for example, are conditional on a university promising to take on scientists after their Wellcome fellowship expires.

The major grants themselves are intended to provide scientists with a stable working environment. Each centre is being seen as a 'honeypot' to attract top-quality scientists and research teams, both from other parts of Britain and from overseas, by offering facilities that neither the universities nor the MRC are able to provide on their own.

Last year's decision to provide £50 million to help establish a centre for genome research in Cambridge has been followed by

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Bridget Ogilvie of the Wellcome Trust tries to follow the dictates of founder Sir Henry Wellcome a half-century after his death.

a grant of £27 million to set up a new centre in Oxford for the study of genetic influences on common diseases, such as diabetes and asthma, £18 million towards a new laboratory at the Institute of Neurology in London, and £4 million for a building near Cam-

US company foundation gets \$400 million

London. The Wellcome Trust will give £270 million (US\$400 million) over the next five years to the Burroughs Wellcome Fund, its US cousin based at the headquarters of the pharmaceutical company Burroughs Wellcome in Research Triangle Park, North Carolina.

The US fund, which was set up in 1951 and which makes competitive awards in the biomedical sciences, is at present a relatively modest enterprise, last year making grants of just under \$6 million. The new money from the British foundation, which comes out of proceeds of the sale of US\$3.2 billion of shares in Wellcome plc last summer (see above), will immediately quadruple the amount of money available for awards, with continued growth for several years.

The size of the grant has surprised many biomedical scientists in Britain, who argue that US scientists already have many sources of funding and that the money might have been better spent on promoting medical research either in Britain or — given the foundation's long-standing interest in tropical diseases — in the developing world.

Bridget Ogilvie, the director of the trust, says that the grant is intended to achieve two objectives: to improve the international

distribution of the foundation's awards and to reflect the US origins of Henry Wellcome, the founder of the pharmaceutical company who left all his shares to the trust when he died in 1936. "We believe that it would be Sir Henry's fervent wish that the trust should make a significant contribution to medical research in the US", says Roger Gibbs, the chairman of the trust.

The US fund has set up a working group of scientists to recommend ways to spend the new money. Officials in North Carolina say that it intends to continue to support areas of research felt to be underfunded, such as experimental drug therapies and parasitology.

In addition, the fund has promised to set up a new fellowship scheme to support British postdoctoral scientists visiting US laboratories for two years, with a further year of support back in Britain. Such a scheme, named after the company's two Nobel laureates in medicine, George Hitchings and Gertrude Elion, has worked in the opposite direction since 1990 through the Fogarty International Center at the US National Institutes of Health, with the Wellcome Trust each year supporting the work of about a dozen US scientists in Britain.

D.D.

bridge to house the European Bioinformatics Institute. Mark Lathrop will be joining the Oxford centre with his team from the Centre d'Etude du Polymorphisme Humain in Paris, and the London institute has already attracted several British scientists.

Other major grants under consideration include applications to support research programmes in cell biology and cell function at the University of Edinburgh, biochemical medicine in Dundee, immunology in Glasgow, developmental biology at UCL and the pathogenesis of infectious diseases at Imperial College, London. Cambridge University is also planning to ask Wellcome for support for a new clinical sciences building.

The research community is generally supportive of the trust's goals, and welcomes the additional funding at a time of job shortages and bleak career prospects at universities. As one scientist puts it, "without the Wellcome money, British medical science would be in big trouble".

But Wellcome has not been totally immune to criticism. Some scientists, for example, complain that, in common with most private charities, it is still run too much as an old boys' club, receiving advice from a relatively small group of individuals within Britain's biomedical research establishment.

Others claim that the trust interprets its funding guidelines more rigidly than is necessary, denying research funds to those who could benefit from them. One scientist complains that he cannot apply for support because his institute has the word 'cancer' in its title, even when the work in question has nothing directly to do with the disease.

Another charge is that the trust, wary of undermining its charitable status, does less than it could to encourage the commercial exploitation of research by the scientists it supports. Unlike bodies such as the Cancer Research Campaign (and the MRC), Wellcome has no programmes to promote the transfer of research results to industry.

Wellcome officials are used to fending off such criticism. They point to the strict system of peer review as proof that awards are based strictly on merit, and they do not apologize for adhering to guidelines contained in the trust deeds.

On the question of commercialization, for example, director Bridget Ogilvie says that the trust is explicitly forbidden from supporting the activities of Wellcome plc. Indeed, some areas of research — such as tropical medicine — have been selected partly because of their neglect by pharmaceutical companies.

Ironically, the biggest problem now facing the trust is how to meet the expectations created by its new wealth. In fact, this year the trust seems unlikely to spend on medical research the £220 million that it now has available. It is the type of problem that other research charities, many of whom are experiencing reduced contributions from donors, would be only too happy to be facing.

David Dickson

UCL uses loan to bet on quality

London. Flushed with the success of its biomedical departments in a recent assessment of research in British universities (see *Nature* **362**, 4; 1993), University College, London (UCL), is to borrow £30 million from the Wellcome Trust to establish a new centre for basic and clinical research.

The university will use the money to buy and refurbish the 87-year-old Cruciform Building, a five-storey red-brick building designed by the eminent Victorian architect Alfred Waterhouse, who was also responsible for the Natural History Museum in South Kensington. The building is owned by the National Health Service and houses the main wards of University College Hospital, which are due to close in July. Although the patients will be moved to the nearby Middlesex Hospital, which also forms part of UCL Medical School, the building is subject to a preservation order.

Under the unusual arrangement, the college plans to pay off the interest-free loan from Wellcome in instalments through future applications to the trust for research support. "In other words, the more successful we are in raising research funds from Wellcome, the earlier we will be able to repay the loan", says Derek Roberts, the provost of UCL.

The trust sees the arrangement as a solution to what its director, Bridget Ogilvie, calls a 'chicken and egg' situation in which universities can find it hard to obtain funds for a major new research building without commitments from scientists to work in them; but without the guarantee of adequate facilities, scientists are reluctant to make such a commitment. UCL scientists hope that the Wellcome loan will strengthen their hand in negotiating with research groups to join the new institute, while other universities such as Edinburgh are considering similar agreements with the trust.

D.D.

Hughes institute displays new home

Washington. The largest private medical research institute in the United States, the Howard Hughes Medical Institute (HHMI) with assets of more than \$7 billion, has moved into a new headquarters and conference centre that provides tangible evidence of stability after years of ferment.

The \$55-million headquarters, near the National Institutes of Health in Bethesda, Maryland, contains offices for an administrative staff of 200 as well as for regular meetings of outside scientists — in particular, the 224 investigators from across the United States that it supports. For years, Hughes investigators have tended to be senior researchers with proven track records, but younger scientists now make up about a third of the total.

The institute will distribute \$270 million in research funds this year and another \$50 million for undergraduate science education. Although HHMI does some work abroad and has plans to expand into the former Soviet Union and eastern and central Europe, only \$5 million of its annual budget is at present spent outside the United States.

Speaking last week at dedication ceremonies, HHMI president Purnell Choppen predicted that the 22-acre campus will become "one of the world's great meeting

places for scientists and investigators" and has been built to meet Hughes's needs for the next century.

The institute was set up by the enigmatic billionaire Howard Hughes in 1953 as a tax shelter, but had funded only modest amounts



The conservatory provides open space under cover.

of research — \$60 million in total — before his death in 1976. In 1985 HHMI's court-appointed trustees sold its main asset, the Hughes Aircraft Corporation, to General Motors for \$5.2 billion, and started a philanthropic programme that has concentrated on supporting a small number of established centres of excellence in five areas of research: genetics, cell biology, neuroscience, immunology and structural biology.

Colin Macilwain

Congress wrestles with electronic highway bill

Washington. A dispute over the role of the US government in operating prototype information networks has been resolved, but a wider conflict in the Senate over which agency will be assigned to lead the effort remains a sticking point for three bills that are central to the Clinton administration's plan to expand investment on such networks.

The proposed legislation would allow federal and state agencies to build research

may prevent full implementation.

Three bills — one in the House of Representatives and two in the Senate — are of particular importance in determining the shape of government support for the so-called information superhighway — a national, high-capacity telecommunications network that the administration believes will strengthen the US economy. The High Speed Performance Computing and High Speed Networking Act, sponsored by Rick Boucher (Democrat, Virginia), chairman of the science subcommittee of the House Science, Space and Technology Committee, is the only one of the three devoted specifically to information technology.

The Boucher bill amends the 1991 High Performance Computing Act to require the Office of Science and Technology Policy (OSTP) to plan and run a five-year programme for networking research. The National Science Foundation (NSF) would take a leading role, spending around half of the \$1.5 billion needed to build network testbeds, subsidize schools and universities to use networks and develop applications.

OSTP director John Gibbons testified two weeks ago that Boucher's bill was "important and forward-looking legislation" but that it contains three flaws. Gibbons says there is no need for a fifth OSTP associate director to run the programme but that the bill should acknowledge the role of the education, energy and defence departments. In addition, he says that restrictive language on when 'testbed' networks must be transferred to the private sector "would complicate an already difficult task".

Boucher concedes that 18 months is an "arbitrary" length of time but says that any compromise must uphold the principle that publicly funded networks should not do commercial work. The issue of a fifth associate directorship may be resolved by assigning responsibility for the information network to someone of lower rank.

After that, the going gets rougher. Two bills in the Senate sponsored by powerful committee chairmen — the Department of Energy National Competitiveness Technology Partnership Act by J. Bennett Johnston (Democrat, Louisiana) and the National Competitiveness Act by Ernest Hollings (Democrat, South Carolina) — each contain sections dealing with the information network. But Democratic leaders would like to merge the two bills. Johnston's gives the Department of Energy a leading role, while Hollings's favours the Commerce Department, so these differences would need to be reconciled before a vote is taken.

Holling's bill is closely aligned to what the administration wants to do, and its provisions on an information highway contain objectives similar to those of Boucher's bill, including rules for the transfer of testbeds to the private sector. It also incorporates a proposal by Senator Bob Kerrey (Democrat, Nebraska) to develop more efficient ways to digitize library books. Johnston's bill specifies a role for Department of Energy laboratories in various aspects of network development and application as part of a \$300-million programme over three years.

Colin Macilwain

Germany names eastern engineer to research post

Munich. Germany today swears in its third research minister this year after Matthias Wissmann was promoted to the transport ministry after only 106 days in office. The appointment of east German Paul Krüger, 42, comes as a major surprise, even to him: "I was shocked [by the offer]", he says, "but thinking it over I very quickly accepted".

A mechanical engineer by profession, Krüger has had a short but very successful political career. He joined the Christian Democrat party (CDU) after the fall of the Berlin Wall in 1989 and became a member of the East German parliament after the first democratic elections in March 1990 and subsequently a member of the new German parliament following reunification. Since then he has been a member of the parliamentary committee on science and technology. He joined the industry and economics committee last year, where he has been active in campaigning for greater support for industry in eastern Germany.

Germany's research ministry has had a turbulent year. In January, a surprise government reshuffle removed Heinz Riesenhuber, Germany's longest serving research minister, after 10 years in the job. He was succeeded by Wissmann, a lawyer whose youth and constituency were more in line with Chancellor Helmut Kohl's expectations of a geographically balanced cabinet (see *Nature* 361, 286; 1993). But Wissmann became Germany's shortest serving research minister when he was offered the transport ministry last week after Günther Krause, an east German, was forced to resign after a series of corruption scandals. Krüger's appointment preserves that geographic balance.

Alison Abbott



From left, Boucher, Johnston and Hollings.

'testbeds' of high-speed information networks and develop computer software to help schools, libraries, universities and government departments use the networks.

Strong lobbying by the telecommunications industry has resulted in a provision that would, 18 months after the bill is enacted, prohibit testbeds from being used for any services that could be provided commercially. This ban meets industry concerns that successful, state-subsidized testbed networks — such as the National Science Foundation (NSF)'s existing NSFNET — undermine the viability of commercial alternatives as well as satisfying those who hold that the government should not operate such services. But academic scientists are worried that a blanket ban would disrupt their work on the networks, and a compromise is being drawn up to meet their concerns by extending the transition and giving agencies greater discretion on its execution.

The provision is designed to ensure that future, multi-agency networks developed under the auspices of the National Research and Education Network (NREN) are swiftly transferred to the private sector in the same way that NSF is now planning to transfer users of NSFNET (see *Nature* 362, 582; 1993). The NSF has said it will provide sufficient funds to enable scientists to continue their work, but the legislation will not guarantee access.

The proposals, which would cost less than \$100 million in their first year, are already part of the president's budget proposal for fiscal year 1994, which starts on 1 October this year. In subsequent years, however, the annual cost would rise to \$400 million, and budgetary constraints

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Paul Krüger

NIH task force to examine 'culture' of discrimination

Washington. Saying that "where there's smoke there's usually fire", the director of the US National Institutes of Health (NIH), Bernadine Healy, has formed a task force to examine racial and sexual discrimination on campus in Bethesda, Maryland.

The task force is Healy's response to recent public demonstrations by two civil rights groups of their unhappiness with NIH's handling of a report by an outside consultant that found evidence to support allegations of discrimination and favourit-

ism by senior managers in the division of acquisitions management (see *Nature* 363, 6; 1993). It is an attempt to erase the negative publicity that NIH has received for its failure to address the problems raised in the seven-month-old report by showing that NIH is concerned about the issue and can move quickly.

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Healy moves quickly.

Speaking to some 200 NIH employees at a rally last week on the lawn in front of her office, Healy said that the report revealed activity that is not acceptable. NIH already has an Office of Equal Opportunity (OEO) to handle complaints from individuals about problems on the job, but the new task force is expected to examine "environmental" issues at NIH that contribute to sexual and racial discrimination at all levels.

The task force will be led by John Diggs, head of the extramural research programme and the highest-ranking African American at NIH, but details relating to its membership, scope and duration have yet to be worked out. Diggs, who is leaving NIH in a month to become vice president for biomedical research at the Association of American Medical Colleges (AAMC), was asked to chair the group only minutes before Healy spent an hour with representatives of the National Association for the Advancement

of Colored People (NAACP) and the NIH chapter of Blacks in Government (BIG). The groups held a press conference two weeks ago to discuss the report and convened last week's lunch-time rally. Vincent Thomas, president of BIG, says that the task force is "a very positive thing" and that its creation sends a signal to NIH managers that "discrimination will not be tolerated". Thomas, who will serve on the panel along with both senior NIH administrators and rank-and-file employees, praised Healy for her willingness to deal with the issue but says that it is too early to predict the outcome of its deliberations. Healy is leaving NIH at the end of June and it could be several months before a successor is named and takes up the post.

Diggs, who had not yet read the consultant's report when Healy tapped him, said that he hopes the task force "will develop strategies to redress grievances" quickly. "A lot can happen if you let a case drag on for seven years", he says, referring to the length of time since the first complaints were brought against managers in the procurement office. At the same time, he says, some employees have failed to follow up their initial complaints with sworn statements and other documentation because they fear reprisals from superiors.

Last week, the NAACP tried to remedy that situation by sponsoring a 'complaints fair' to collect additional evidence of discrimination on the job. NIH employees were encouraged to bring evidence documenting their complaints and were then matched with local attorneys who have agreed to represent them without fee. OEO director Diane Armstrong says that the complaints will be processed as quickly as possible, taking advantage of a recent fourfold increase in her staff.

In a related matter, an OEO advisory committee of black employees is petitioning NIH to block the planned move of OEO offices from the main campus to satellite offices several miles away. The petitions claim that the move, which is being made next month to bring Armstrong's staff together on the same floor, would make it harder for employees to present their complaints and would require them to receive permission to leave the campus from the same supervisors who might be the subject of their complaint.

Armstrong says that any employee may visit her office but that permission must be obtained whenever someone leaves the work place. "I don't see our new office as a barrier", she says. "In fact, some people have told me that they prefer us to be in a less conspicuous location." **Jeffrey Mervis**

Changes urged in US government hiring practices

Washington. With the current recession making public service an attractive alternative to the private sector, a new report on what the US government must do to attract and retain the best scientific talent addresses a problem that — at least for now — does not exist. But if the time to fix a roof is when the sun is shining, then the report by the National Academy of Sciences provides useful advice on what the federal government can do to avoid the inevitable next flood of complaints.

Three important improvements would be delegating hiring authority to those at the work site, easing restrictions on — although not necessarily increasing — starting salaries and making increases contingent on performance ratings. The changes, according to the report by the academy's National Research Council, would make the government more able to compete with industry for qualified scientists.

Although economic conditions have deteriorated since the study was started two years ago and US President Bill Clinton has proposed a one-year freeze on federal salaries, money is not the key to better recruiting and retention practices, says the academy's Alan Fechter. "We are talking about creating tools for good management", Fechter says. "If hiring takes less time and generates less paper work, then we've accomplished something."

Several US agencies have conducted demonstration projects to test such management changes. At the National Institute of Standards and Technology (NIST), project chief Allen Cassidy says that streamlined procedures that were introduced in 1988 shortened the hiring process and identified more highly qualified people. "We don't have to match industry dollar for dollar, since the work here is interesting", he says. "We just need to close the gap enough that people feel they can afford to move." NIST maintained a level personnel budget during its project by hiring fewer people than it could have done.

The academy's report recommends a process in which agencies are allowed to design appropriate changes after consulting other departments and in conjunction with the Office of Science and Technology Policy and the Office of Personnel Management. In the longer term, the report suggests that the government should develop a database to monitor the way in which policy changes affect the work force, evaluate its policies every four years and create a category specifically for scientists and engineers. Both agencies say that the report is being given serious consideration.

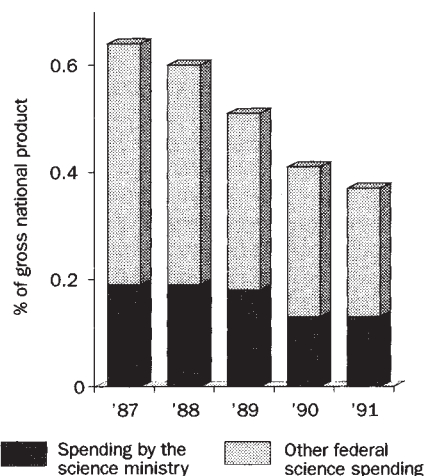
Jenna Roberts

Brazil's science budget looks good — on paper

São Paulo. Brazil's Ministry of Science and Technology has been allocated the equivalent of nearly US\$1 billion this year, but that record level of spending masks accounting tricks and a continuing decline in the proportion of the gross national product being invested in science.

For Brazilian scientists, the government's 1993 budget — which was delayed several months because of the political crisis that led to the impeachment of the president, Fernando Collor de Mello — sends a mixed message. Although it is about 15 per cent

A shrinking commitment to science



larger than last year, it offers no guarantees that the money will actually be spent on science. Last year, for example, nearly half of the budget was transferred to other sectors or swallowed up by inflation, and only recently the programme that funds graduate students reported that it was unable to pay the monthly stipends in April because of delays in receiving its allocation.

The Brazilian Society for the Progress of Science believes that the budget is a reasonable one, given the country's economic difficulties and the commitment of the new president, Itamar Franco, to invest in social programmes. But science minister José Israel Vargas is rumoured to be contemplating resignation out of frustration.

Vargas's priority this year is to finish the so-called 'strategic projects' he inherited and to reverse three years of budgetary neglect. For example, the synchrotron laboratory near Campinas, under construction since 1987, was supposed to be ready last year; under the new budget it will be completed by the end of 1994.

Computer science is another area that Vargas wants to encourage. The budget includes more money for the National Laboratory for Scientific Computing, which hopes by the end of next year to move from Rio de Janeiro to a larger facility with a supercomputer in Petrópolis. Resources for a Centre for Informatics Technology were increased by 45 per cent, and a programme called Softex-2000 is intended to create a thousand companies by the end of the century producing software for export.

Brazil's burgeoning space programme will also receive a boost in the current budget. In February the first indigenous Brazilian satellite was launched by a rocket designed by a US company, Orbital Sciences Corporation (see *Nature* 360, 290; 1992), and there are plans for its successor, also a data-collecting satellite, to be launched by a domestic rocket from the new space centre at Alcântara.

Its success depends in part on Brazil's ability to overcome the suspicions of industrial countries that the rocket is designed to launch ballistic missiles as well as domestic satellites. Those countries have imposed a boycott on exports to Brazil on the grounds that the programme violates an international regime to limit the proliferation of missiles capable of delivering nuclear weapons.

Last week, Franco took steps to calm those fears by announcing the creation of a space agency independent of the military. The new agency will have its own budget and hire its own staff, unlike its predecessor, which coordinated the activities of the science ministry and the Air Force but which was linked to the general staff of the armed forces. The opening paragraph in the law that creates the new Brazilian Space Agency declares its civilian status.

Vargas is also hoping to put additional resources into a joint project with China to develop two remote-sensing satellites. Little progress has been made since the agreement was signed in 1988, and Brazil has not yet put up its US\$20-million share of the US\$56-million project. The civilian National Institute for Space Research is hoping that a new drought in the country's north-eastern region will spur investment in its Centre for Weather Prediction and Climate Studies, another project on Vargas's list.

Other ministries, state companies and banks spend even more on science and technology than does the science ministry. But the budgets of the other ministries have been falling at a greater rate than the science ministry's as a proportion of the country's gross national product, and this year's budget seems likely to continue that trend.

Ricardo Bonalume

NEWS IN BRIEF

Tokyo. Despite persistent charges of mismanagement and financial irregularities at the top of the World Health Organization (WHO), Japan's Hiroshi Nakajima was reelected last week to a second five-year term as its head. In January, Nakajima survived an attempt by his former deputy director, Mohammed Abdelmoumene of Algeria to unseat him (see *Nature* 361, 290; 1993), and his reappointment has been confirmed by the general assembly by a vote of 93 to 58, the narrowest margin in the organization's history. In the interim, a report provided evidence of questionable WHO contracts being awarded to organizations with close ties to some of those who voted for Nakajima. Japanese government officials expressed relief at last week's vote, but admit the result is "not entirely satisfying". Nakajima is the first Japanese to lead a UN agency. **D.S.**

Washington. Roger Herdman, a former medical researcher and veteran health policy analyst, has been elected director of the US Office of Technology Assessment (OTA), succeeding John Gibbons, who in January became science adviser to US President Bill Clinton. Herdman joined OTA in 1984 as assistant director of its division of health and life sciences, and since January has been acting director of the congressional agency. Previously he was a vice president of Memorial Sloan-Kettering Cancer Center in New York City and director of public health for the state of New York. He holds an MD degree from Yale University and has been a professor of paediatrics, specializing in children's kidney diseases, at Albany Medical College. Herdman led one of three divisions at OTA, which has an annual budget of \$21 million to conduct studies for Congress on a range of technology issues. **J.M.**

Washington. Marc Brodsky, a staff scientist at the IBM Thomas J. Watson Research Center, has been chosen as director and chief executive officer of the American Institute of Physics (AIP). Brodsky



Marc Brodsky

replaces Kenneth Ford, who will retire on 31 October after nearly seven years as the head of AIP. Brodsky, a physicist who has worked on the technology of semiconductors, joined IBM in 1968 and was head of technical planning for the company's research division before spending last year studying critical manufacturing technologies for the US Commerce Department. AIP is composed of 10 member societies with a combined membership of about 100,000.

J.M.

FDA panel sees problems with labelling of milk hormone

Washington. An advisory committee to the US Food and Drug Administration (FDA) appears to oppose the mandatory labelling of food products derived from dairy cows treated with a recombinant form of bovine growth hormone (rbGH), but Congress may take up the issue and it seems likely that opponents of rbGH will wage a campaign at the grocery store.

The committee last week held two days of public hearings on the question, which involves a hormone used to boost milk production in dairy cows. Although the committee stopped short of making formal recommendations to FDA, more than two-thirds of its members spoke out against requiring mandatory labelling and said that an alternative would be voluntary labelling of products from cows not treated with the hormone provided FDA could authenticate such claims.

"I don't know that I got a real consensus out of it [the meeting]", says Gerald Guest, director of FDA's Center for Veterinary Medicine, "but I think people recognize the difficulty if you did label. I heard most people saying, 'Well, even if the government doesn't require it, voluntary labelling would be OK', and I think that's true."

The committee heard testimony from more than 50 individuals representing animal rights groups, biotechnology companies, consumer and citizen action groups, dairy farmers, environmentalists, members of Congress, physicians, trade associations and veterinarians. Although the debate, often heated, centred on whether foods derived from dairy cows supplemented with rbGH should be labelled as such, and if so why, the issue at times became blurred by those who oppose the use of rbGH for reasons outside the purview of FDA.

Opponents say that special labelling is unwarranted and unprecedented and would have considerable implications for food labelling requirements generally. They argue that FDA does not have the authority to require labelling given that bGH occurs naturally in milk, that studies have found no discernible difference in trace levels of bGH before and after supplementation and that milk from supplemented cows and untreated cows is indistinguishable in terms of composition, quality, taste and texture. The situation is compounded by the practice of pooling milk from farms and by the lack of a practical and accurate method of distinguishing between rbGH and bGH that occurs normally in milk.

In 1992, the General Accounting Office issued a report suggesting that, although rbGH does not appear to represent a direct human food safety risk, FDA should with-

hold approval until the question of indirect human food safety risks is resolved (see *Nature* 358, 614; 1992). An FDA veterinary medicine advisory committee last March declared that a statistically significant increase in mastitis (inflammation of the udder) in cows treated with rbGH was a "manageable risk" that would not lead to an increase in antibiotic residues in milk.

Proponents of labelling believe that FDA has underplayed the mastitis issue and argue that a "manageable" risk is a risk nonetheless and that consumer surveys indicate that people want to be given the choice of whether or not to purchase such products. "If the FDA gives the green light to unlabelled bGH products", says Jeremy Rifkin of the Pure Food Campaign, "we intend to defeat these products at the marketplace" with a sustained consumer boycott.

Although federal regulations prohibit the FDA from saying when and if rbGH might be approved, Guest did venture that he felt "it's very much a public policy issue more than a scientific issue now" and that Con-

gress might want to get involved.

Bovine growth hormone (also called bovine somatotropin, or bST) is a protein hormone produced in the pituitary gland of cattle that plays an important role in regulating the metabolism of carbohydrates, fats and proteins. It is one of 30 hormones known to be normally present in milk and is not biologically active in humans. Although it was shown in the 1930s that milk production in dairy cows could be increased by giving cows supplementary bGH injections, its commercial use was prevented by the limited supply of purified bGH derived from pituitary glands. With the advent of biotechnology, large quantities of biosynthetic bGH can now be produced using recombinant DNA technology and used to raise milk yields by an average of 10–15 per cent.

Four companies — American Cyanamid, Elanco (a division of Eli Lilly & Co.), Monsanto and Upjohn — are seeking FDA approval for their particular recombinant form of bGH. These products are either similar or identical (in the case of Upjohn's product, Somavubove) to the naturally occurring bGH variant containing 191 amino acids. The other three products differ in the amino acids that are substituted for the terminal alanine residue.

Diane Gershon

Italian court wrestles with cold fusion suit

Munich. An Italian judge trying to decide if cold-fusion advocates Stanley Pons and Martin Fleischmann were libelled by the daily newspaper *La Repubblica* has called in a technical adviser to help him understand the scientific aspects of their work, which created an international sensation when it was announced in 1989. The use of a technical adviser, a new procedure for Italy, is one of several novel features of the case, which is being tried in a country with no legal definition of scientific fraud.

In October 1991, Italian journalist Giovanni Pacci is alleged to have libelled British chemists Fleischmann and Pons by referring to them as scientific frauds in a strongly worded review of a new translation of Axel Kahn's book *False Prophets*. Pacci

made an indirect reference to a group of Italian scientists working on cold fusion and compared scientific frauds in general to 'fornicating priests' because of their betrayal of science as a 'temple of truth'.

Although they were not mentioned by name, Giuliano Preparata, Tullio Bressani and Emilio Del Giudice of the Institute of Physics in Milan believed that they were identifiable and requested a retraction. Instead *La Repubblica* published their letters along with a defiant commentary from Pacci. As a result, the Italians joined Fleischmann and Pons at the beginning of last year in suing the influential newspaper for IL8 billion (US\$5 million), an unusual action in a country where such lawsuits are rare.

The case itself does not address the issue of cold fusion, but rather whether the scientists can justly be accused of fraud. Even so, the judge decided to obtain an independent assessment of the 105 'pieces of scientific evidence' submitted by the plaintiffs and appointed Giovanni Licheri, a physical chemist from the University of Cagliari in Sardinia, to tell him whether the data could have been fraudulently reported.

Licheri has six months to form an opinion on the scientific validity of the work, a point of rancorous debate for two years after the initial announcement in March 1989. A first meeting took place on 28 April involving Licheri, the five scientists who filed the lawsuit and Douglas Morrison of CERN, who was chosen as a scientific adviser by *La Repubblica*. Morrison publishes an electronic mail newsletter that takes a sceptical view of cold fusion.

Alison Abbott

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

**Pons and Fleischmann,
right, with 1989 model.**

Problems with Galileo

SIR — "Does the Earth go round the Sun in any but a relative sense?" asked a leading article in *Nature*¹, and gave this answer: "Prescient Galileo was probably too good a scientist to have committed himself to an absolute view on such a question." But in the preface of his book *Dialogue Concerning the Two Chief World Systems — Ptolemaic and Copernican*, Galileo explicitly stated: "The celestial phenomena will be examined, strengthening the Copernican hypothesis until it might seem that this must triumph absolutely"². The Vatican Inquisition declared this to be the one and only reason for Galileo's condemnation. Had Galileo not championed the "absolute triumph" of the Copernican hypothesis, he would not have been persecuted.

Thus the Galileo story remains more of a present problem than your leading article supposes. Evidently, the writer has been influenced by the ideas of Einstein, who claimed that all coordinate systems (both inertial and non-inertial) are equivalent. According to Einstein, "the two sentences, 'the sun is at rest and the earth moves' or 'the sun moves and the earth is at rest'... can be used with equal justification"³.

It has taken the Catholic Church several centuries, plus twelve years of scrutiny by a special commission of inquiry (appointed by the Pope in 1979), but they have finally caught up with Copernicus and Galileo. One may wonder how long it would take them to catch up with Einstein!

The Vatican commission correctly remarked that Galileo was mistaken in his belief that he had established a proof of the Copernican theory. The evidence that Galileo produced was indeed inconclusive. The commission also remarked, again correctly, that a universally accepted verification of the Copernican theory was obtained only nearly a century after Galileo's trial in the form of the annual stellar aberration, discovered by Bradley in the 1720s⁴.

Further indisputable proof came in the 1830s with the discovery of the annual stellar parallax⁵, an effect that was actually predicted by the heliocentric theory, and by this theory alone. All other theories make predictions at variance with the observed stellar parallax and aberration. It was generally agreed in the nineteenth century that what the observed parallax and aberration show is that the Earth *really* moves with respect to the Sun, and to the Sun alone (or, more accurately, the centre of gravity of the Solar System). In the nineteenth century, stellar parallax and aberration were universally regarded as conclusive proofs of the physical super-

iority of the heliocentric system over the systems of, say, the Earth, or Mars, or Saturn, or Ganymede.

If one wants to advocate the equivalence of all systems, one would have to re-interpret stellar parallax and aberration in terms of this hypothesis. How is this done? To the best of our knowledge, it has never been done — a rather curious and serious omission.

In our experience, attempts to publicize these issues in both scientific and public forums have been thwarted by the non-religious Inquisitions of the twentieth century. Thus the Galileo affair has more present significance than the *Nature* leading article has recognized.

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2. Galilei, G. *Dialogue Concerning the Two Chief World Systems — Ptolemaic and Copernican*, trans. by S. Drake (University of California Press, Berkeley, 1952).
3. Einstein, A. & Infeld, L. *The Evolution of Physics*, 224 (Cambridge University Press, 1938).
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5. Bessel, F.W. *Astron. Nach.* **16**, 65–96 (1838).

Keeping warm

SIR — August Reader (*sic*) suggests that DREADCO biochemists should turn their attention away from metabolic stimulants and consider ancient transcendental techniques for generating 'magical heat' as a means to prevent hypothermia (*Nature* **361**, 200; 1993). Unfortunately, many of these trance-like conditions result in hypometabolism (reduced heat production) and an increase in skin blood flow that will increase heat loss from the body.

In hot climates, the reduction in metabolic rate and the increase in heat loss would help thermoregulation and save on air-conditioning, but in cold environments would lead to hypothermia, or increased heating costs — quite the opposite of DREADCO's research objective. Moreover, Reader's suggestion that DREADCO biochemists should look for adrenergic blocking agents would be counter-productive as these agents would inhibit sympathetically mediated thermogenesis, thereby depriving the individual of a valuable source of body heat.

In most mammals, particularly neonates, sympathetic activation of brown adipose tissue (BAT) is the main source of cold-induced and diet-induced thermogenesis. Thus, DREADCO biochem-

ists were on the right track when looking for agents to 'burn off' food calories, but if they had consulted the DREADCO pharmacologists they would have found out that BAT-selective agonists (known as β_3 -agonists) have already been developed for use as thermogenic antiobesity agents. Several pharmaceutical companies have such agonists in development, although it seems unlikely that they will be marketed as an aid to reducing heating costs, particularly as food energy is more expensive than gas or electricity.

Incidentally, you and other editors may have recently received promotional material for a revolutionary new type of clothing (CLOtherapy) that claims to work by letting "key areas of your brown fat sense and respond to cool outside temperatures", and which "by working out your brown fat can help you control your body fat". Given that most human BAT is found around the heart, kidneys and major arteries, one wonders about the invasive nature of this apparel. On reading this material I was convinced that DREADCO scientists had ventured into the 'rag trade', but on reflection, they clearly would not have produced anything so daft.

Michael J. Stock

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Not exclusive

SIR — Your article referring to Frank Grosveld's work on regulation of gene expression (*Nature* **361**, 572; 1993) says that "Therexsys will have an exclusive licence to the intellectual property, both existing and in future, arising from this work".

The Medical Research Council (MRC) has not granted such all-encompassing rights. The council's policy is to exploit its intellectual property as widely as possible in the interest of healthcare improvement. As in other cases, MRC has therefore granted to Therexsys exclusive rights to specific past and current work within a defined field. The range of rights licensed to Therexsys is, we believe, that needed to allow successful exploitation of the specific research findings and development of Therexsys. Work in Grosveld's laboratory has other applications, outside this field, and MRC has licensed and will continue to license these to organizations capable of constructive industrial exploitation.

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Has Duesberg a right of reply?

Dr Peter Duesberg, the virologist-turned-campaigner, is wrongly using tendentious arguments to confuse understanding of AIDS and those in danger of contracting the disease. He should stop.

WHAT is to be thought of a science journal that publishes attacks on the opinions of a scientist, but which never (or hardly ever) publishes his replies? On the face of things, this is a serious breach of journalistic ethics — and it would be legally prevented by the legislation on the press perpetually being considered by the British House of Commons. Yet that is how *Nature* has behaved to Dr Peter Duesberg, the virologist from Berkeley who is identified with the view that HIV does not cause AIDS. How can such intolerance be justified?

What follows is an explanation. But first, an essential part of the tale is that Duesberg is a molecular virologist of distinction; his last major publication in *Nature* (304, 219; 1983) was a classic of its kind. He is also a person of intelligence and good humour. Moreover, there are elements in his position on the causation of AIDS that are not nearly as perverse as they are often represented.

Early on, Duesberg raised some cogent questions. For example, why, if HIV is an infectious agent and also the cause of AIDS, is it so difficult to recover virus from the particular T cells (CD4 cells) that are supposedly its targets? And why do morbidity and mortality among those with haemophilia infected by contaminated blood products appear to be so much less than among those who have acquired HIV by, say, homosexual sexual intercourse?

These questions were proper questions, if not the only ones that may legitimately be asked about AIDS as a disease. Why, for example, is otherwise rare Kaposi's sarcoma so commonly one of the consequences of infection by HIV (and why is it uncommon among infected haemophiliacs)? Why is diarrhoea often one of the first symptoms of overt AIDS, and what can be done to prevent its debilitating effects? Why is the "incubation period" so long and variable? A full understanding of the pathogenesis of AIDS would answer all these questions.

So why scorn Duesberg's demand of the research community for answers to his proper questions? Part of the explanation is that Duesberg has not been asking questions, or raising questions he believes should be answered, but has been making demands and implying (but sometimes saying outright) to colleagues, "Unless you can answer this, and right now, your belief that HIV causes AIDS is wrong". It is as if a person were to have told Schrödinger in 1926, "Unless you can calculate the spectrum of lithium hydride, quantum mechanics is a pack of lies". (Interestingly, that deceptively simple question is

only now being answered.)

Unanswerable rhetorical questions are the stock-in-trade of undergraduate debating societies. In the grown-up world, the obligation to answer even well-posed questions is a function of circumstances. True, good theories (*pace* Popper) are falsifiable theories, and a single falsification will bring a good theory crashing down. But unanswered questions are not falsifications; rather, they should be the stimulants of further research. Whether researchers divert effort from current preoccupations to answer other people's questions properly depends on their personal judgement of the cogency and relevance of the questions and even on the motives of the questioner.

Duesberg has forfeited the right to expect answers by his rhetorical technique. Questions left unanswered for more than about ten minutes he takes as further proof that HIV is not the cause of AIDS. Evidence that contradicts his alternative drug hypothesis is on the other hand brushed aside. Thus Duesberg's reply to A. J. Pinching's question why, if transfusions of Factor VIII are the true cause of AIDS among haemophiliacs, do patients uninfected by HIV never contract AIDS, is tantamount to an assertion that the patients must have been selected with bias or that the data must be false (see *Nature* 350, 10; 1991).

For what it is worth, Duesberg's remark about the difficulty of recovering virus from T cells is linked with the question of the incubation period; both will probably be answered by studies provoked by the recent discovery of large amounts of virus in the lymph nodes of infected people quite early in the incubation period (see *Nature* 362, 355–359; 1993). The difference between the incidence of AIDS among haemophiliacs and homosexual men is more apparent than real (see, for example, C. Lee *et al.* *Br. med. J.* 303, 1093; 1991) and is accounted for by the difference of age distribution between the two groups. (The younger a person, the slower the progression to AIDS.) The rhetorical question why infected haemophiliacs rarely develop Kaposi's sarcoma is logically no more disconcerting than the question why this rare condition is so common among homosexual men with AIDS.

Rhetorical techniques such as these are only barely forgivable among otherwise friendly colleagues; people's patience with travesties of science is understandably thin. Duesberg has made his debating technique thoroughly intolerable by advertising his position to the AIDS community, thus giv-

ing many infected people the belief that HIV infection is not in itself the calamity it is likely to prove.

Nature's most recent refusal of an intended publication by Duesberg was of his response to an article on the "drug hypothesis" by M. Ascher *et al.* (362, 103; 1993), which used data on a group of more than 1,000 men recruited in 1984 in San Francisco to show no correlation between the incidence of AIDS and previous drug-taking. The likelihood of developing AIDS was found to be 1.56 times as great among heavy drug-users as among those using them more moderately or not at all, while the chance of HIV infection was 1.43 times as great in the first group as in the second.

Duesberg's unpublished reply, on the basis of what is most charitably called a misreading of the text, asserts that the authors must have fabricated some of their data. He goes on to claim that the authors' argument supports his view, not theirs. He quarrels with an imagined failure to collect data on drug use after recruitment and claims that a two-year study cannot test the hypothesis of drug toxicity requiring ten years for its effects to become overt. (Readers seeking further information will find the essence of Duesberg's reply, provoked by a report by Ascher *et al.*, in *The Lancet* 341, 958; 1993).

Interestingly, as a referee of his unpublished letter to *Nature* has pointed out, some of the most telling evidence against Duesberg's position arises from the recent Anglo-French study of the use of AZT as a prophylactic of AIDS in people infected by HIV (*The Lancet* 341, 889–990; 1993). The negative finding of that study is also, incidentally, evidence that AZT is not the danger-drug that Duesberg has claimed. There is no mention of that awkward circumstance in his intended reply to Ascher *et al.*

The truth is that a person's "right of reply" may conflict with a journal's obligations to its readers to provide them with authentic information. Whatever Duesberg's friends say, the right of reply must be modulated by its content.

Duesberg will not be alone in protesting that this is merely a recipe for suppressing challenges to received wisdom. So it can be. But *Nature* will not so use it. Instead, what Duesberg continues to say about the causation of AIDS will be reported in the general interest. When he offers a text for publication that can be authenticated, it will if possible be published — not least in the hope and expectation that his next offering will be an admission of recent error. **John Maddox**

Light genes will out

Peter J. Herring

THE anglerfishes (ceratioids) and flash-light fishes (anomalopids) are strange bedfellows. The anglerfishes are found in deep seas throughout the world, and are lurking predators, whereas the flash-light fishes inhabit shallow waters in the tropics, and are active plankton feeders. Yet the two groups have in common the possession of unculturable luminous bacteria, and the general assumption has been that these symbionts are minor variants of known species. Analysis of the symbiont 16S ribosomal RNA genes, reported by Haygood and Distel on page 154 of this issue¹, now demonstrates just how wide of the mark this assumption was.

Luminous bacteria occur in odd places. Robert Boyle encountered "a luminous neck of veal" and undertook the earliest scientific study of them. Their nocturnal appearance in nineteenth-century dissecting rooms led to some interesting, if eccentric, interpretations of the mental history of the corpses, but it is only in the past 70 years that bioluminescent symbionts have been recognized in certain fish, squid and tunicates. Most are extracellular and culturable, and are indistinguishable from 'free-living' species of *Photobacterium*. Almost any sample of sea water contains some luminous bacteria. *Photobacterium leiognathi*, *P. (Vibrio) fischeri*, and several *Vibrio* species occur in shallow water; *P. phosphoreum* is typical of deep water. Symbionts in deep- and shallow-living fishes mirror this pattern. In most of these fishes the light organ connects with some part of the gut. Luminous bacteria are present in gut flora and the acquisition of symbionts by successive generations of host thus presents no practical difficulties, other than the selection of the 'right' species.

The luminous bacteria of ceratioids and anomalopids have stubbornly resisted all attempts at culture. Efforts to relate them to culturable bacteria first centred on the luciferase kinetics², but the real indication that the anomalopid bacteria were special came from a comparison of the luciferase (*luxA*) genes of *Kryptophanaron alfredi* with those of eight known species of luminous bacteria³. This suggested that the symbiont was a new species related to *V. harveyi*, assuming that the evolution of the *lux* gene mirrored the genome as a whole. This was reinforced by analysis of

restriction fragment length polymorphisms, of *lux* and 16S rRNA genes⁴.

Haygood and Distel's new findings show that the symbionts do not belong in any known taxa, but nevertheless share phylogenetic origins with both the *Photobacterium* symbionts and the free-living species of *Vibrio*. They also emphasize that the ceratioid and anomalopid symbionts form separate



Lurking in the murky depths — the anglerfish *Melanocetus johnsoni* displays its luminous lure. (Institute of Oceanographic Sciences.)

monophyletic groups. The wide divergence between the symbionts of different ceratioid families and anomalopid genera is another surprise. In marked contrast, facultative *Photobacterium* symbionts, from hosts in different orders of fishes, are indistinguishable. Obligate symbiosis here seems to equal genetic variety.

The results prompt all sorts of questions. Evolutionary biologists will want to know whether the molecular phylogeny of the symbionts matches that of their hosts. Is the divergence between the symbionts of the anglerfish *Cryptopsaras* and *Melanocetus* typical of the seven other families of luminous ceratioids? Does each have its own 'species' of symbiont? Are the symbionts in the lure and caruncle (a posterior luminous tubercle) of *Cryptopsaras* the same? How divergent are other luminous bacterioids (for instance those in the squid *Heteroteuthis* and the tunicate *Pyrosoma*)? Ecologists and physiologists will wonder, like me, where the bacteria

come from to reinfect successive generations of host. We know they can leak from the host into the surrounding sea water, but do they survive there? Adult and juvenile anglerfishes live at different depths; how does a larval female acquire the right bacterium, even if it is leaked by the adult, if the adult lives several hundred metres deeper?

Rearing generations of captive anglerfish is a long way off, though the anomalopids can be maintained in public aquaria and can perhaps soon be reared there too. Strangely, some specimens lose their bacteria and become dark; they are not then reinfected from other individuals. In contrast, the shallow-living squid *Euprymna morsei* does acquire its luminous symbionts (*P. fischeri*) from the surrounding water, but will accept only certain strains, which induce remarkable morphogenetic changes⁵. Capture of the 'right' strain of symbiont triggers anatomical changes in the young squid. This is particularly evident in the regression of ciliated epidermal processes, whose function is to assist the transfer of the bacterium from the sea water to the waiting light organ. Given the specificity of the anomalopid and ceratioid symbioses shown by Haygood and Distel, the task faced by a newly hatched larva (recognizing and capturing the right symbiont) makes finding a needle in a haystack seem a wholly trivial problem.

Other animals with obligate symbionts face similar problems. The chemoautotrophic bacteria of hydrothermal vents and cold seeps present interesting parallels, particularly among the bivalves⁶ and worms. Their symbionts may be acquired from the sea water, as postulated for the sessile vestimentiferan worms *Riftia* and *Ridgeia*, whose adults lack a gut and which have planktonic larvae. Bacteria are absent from the early stages, which must then acquire the symbionts (a single unculturable strain not far removed from *Vibrio*⁷). *Ridgeia* larvae have a mouth and a functional ciliated gut. The 'right' symbiont is acquired by endocytosis from the gut, which then regresses⁸. The morphogenetic consequences of

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symbiont recognition and acquisition are analogous to those in young *Euprymna*⁵.

If ceratioids and anomalopids do indeed find their symbionts in the water around them (and there is still no proof of this), we too should be able to find them. 16S rRNA gene techniques have already shown how great is the diversity of the bacterioplankton, and how small the subset of marine microbes exposed by culture methods⁹. They should also

be able to determine whether the ceratioid and anomalopid symbionts are present in the sea and available to potential hosts. The odds on finding the needle improve substantially if it is known to be in the haystack. □

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MAGMA GENESIS

Melts caught in the act

Don Elthon

GEOLOGISTS rarely get an unadulterated view of melts from the Earth's interior — changing pressure, temperature and chemical interactions with overlying rocks leave their indelible mark on rising samples. That is why Sobolev and Shimizu's description¹ (on page 151 of this issue) of a rare mantle melt, one of the source materials for mid-ocean ridges, is so exciting.

The series of submarine volcanic mountain ranges that comprise the mid-ocean ridges are the most prominent (if unseen) geological features on Earth. Although it is clear that these immense volcanic ridges result from melting of the Earth's interior 20–100 km beneath the sea floor, the exact nature of these melting processes has long been the subject of intense debate. The lanthanide-series rare-earth elements, which occur as trace elements in oceanic rocks, have been central to this issue. Sobolev and Shimizu report rare-earth data for a melt inclusion trapped within an olivine crystal from one of the largest mid-ocean ridges, the Mid-Atlantic Ridge (see micrograph). The remarkable feature of this melt inclusion is the extremely low abundance of lanthanide elements when compared with other mid-ocean-ridge magmas.

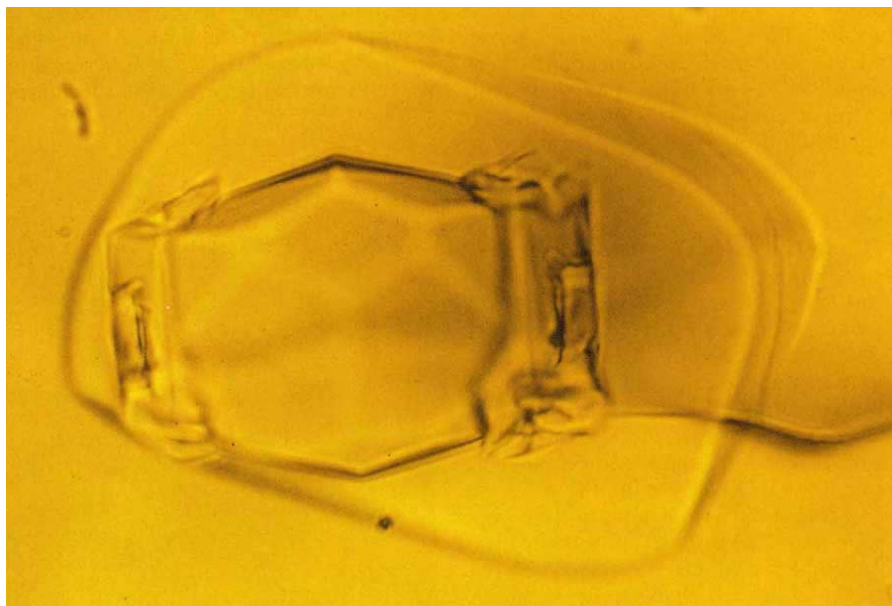
This observation is a critical piece of evidence in a rapidly evolving understanding of how melting and melt segregation occurs within the Earth. Until the mid-1980s, most earth scientists had concluded that melting occurs under batch equilibrium conditions, where magmas produced by melting remain in equilibrium with the residual minerals (magnesian silicates known as pyroxene, olivine and garnet) until melting ceases when 10–25 per cent of the material is liquid; after which the magma ascends to the surface. Recent developments^{2–4} have led most specialists to conclude, instead, that fractional melting (or a variation known as continuous melting) occurs; melt produced under these conditions is separated from the residual

minerals at the time that it forms or shortly thereafter. With fractional melting, a total of 10–25 per cent liquid might still be produced during melting under mid-ocean ridges, but the liquid

ing evidence that they have discovered one of these late melts is the amount of cerium in their sample, which is only 0.09 parts per million, as small as anything expected to result from fractional melting. For comparison, liquids produced by equilibrium melting of the mantle typically have cerium levels of 2.4 parts per million or more⁵.

How did Sobolev and Shimizu find their melt? Magmas erupted along mid-ocean ridges are quenched by contact with sea water and develop thin glass outer rinds, which often contain crystals of olivine or plagioclase with microscopic melt inclusions inside. Tens of thousands of these glass rinds have been analysed over the past 20 years and their range of compositions is well known. Many of the melt inclusions have also been studied and they often have unusual compositions, different from those of the rinds.

It is curious, however, that none of these tens of thousands of glass rinds has



Mantle geology encapsulated: the melt inclusion, 150 μm across, found in an olivine crystal in a basalt dredged from the Mid-Atlantic Ridge at 9° N and described in this issue by Sobolev and Shimizu¹. Within the inclusion is a well faceted crystal of mantle orthopyroxene, edged by feathery clinopyroxene grown during quenching.

will be separated from the residual minerals whenever approximately 1 per cent of melt has formed.

The emerging consensus for melting beneath mid-ocean ridges involves the onset of fractional melting at about 100 km depth, in which small volumes of melts are episodically removed, taking with them the elements least compatible with the residual minerals. When further melting occurs, both the residual minerals and the extracted liquids have progressively lower contents of the rare-earth elements. The later increments of melt produced are expected to have minimal amounts of rare-earth elements.

For Sobolev and Shimizu, the clinch-

been found to contain melts with very low rare-earth abundances, until Sobolev and Shimizu's study. If these highly distinctive melts are routinely produced during melting, where are they? The answer is that the individual small volumes produced at different stages generally accumulate and are intermixed together, either during ascent or in magma chambers just below the sea floor.

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This accumulation and intermixing eliminates the extreme chemical variations that are produced during fractional melting, with the result that the process is hard to distinguish from batch melting. The most likely way to preserve the extreme chemical variations is for samples of these melts to become trapped within crystals as melt inclusions; the encapsulating crystal prohibits accumulation and intermixing. This is exactly what Sobolev and Shimizu have benefited from.

Good fortune, then, and working in

one of the few laboratories worldwide with the capacity to analyse microscopic parts of minerals with parts-per-billion resolution have given Sobolev and Shimizu this rare prize. Capitalizing on it — finding how the melts formed, how they were trapped, where in the melt column they formed, and how variable they are — will keep the authors and their colleagues busy for many years. □

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day cage which screened the lamp's radio emissions. Disturbance by such sources might be one reason why experiments on the magnetic sense have proved variable and difficult to replicate — so, no doubt, lights will be going out in laboratories around the world.

Identifying the receptors of navigational cues is one thing. Understanding how these cues are integrated to make complex navigational decisions is quite another. Here, studies of homing pigeons have figured prominently, with inferences being derived mainly from observations of homeward tendencies in 'vanishing bearings' at release sites remote from the home loft. Yet pigeons do not usually vanish towards home (except in Italy): the homeward tendency has to be extracted statistically from a welter of influences that combine to affect each bird's orientational decision.

Perhaps most mysterious (and certainly frustrating) are consistent site-dependent directional biases. Precise optical triangulation techniques now show that at some sites there are additional biases in initial orientation long

NAVIGATION

Homing mechanisms in sight

Tim Guilford

STUDY of the impressive 'homing' abilities shown by some animals¹ is alive with unresolved issues. Among them are the elusive nature of the 'magnetic sense' and the notorious site-dependent orientational biases of homing pigeons. But we might not have to wait too long for their resolution, for reports from a conference* last month made plain that substantial progress has been made on both counts.

There have been many claims of magnetoreception in a range of animal taxa (as reviewed by W. Wiltschko, Frankfurt University), yet there is scepticism about some of them because responses are variable and the receptors concerned have yet to be identified. It seems, however, that the search for two kinds of receptor, one based on biogenic magnetite (Fe_3O_4) crystals, the other on photoreceptors, is again hotting up. Single-unit recording in the trout trigeminal nerve has revealed neurons that respond to changes in magnetic intensity; moreover, branches of the nerve innervate the ethmoid tissue, which, in related salmonids at least, contains magnetite apparently suitable for magnetoreception (M. Walker, Auckland University). These results are similar to those found in the bobolink, a migratory bird². Meanwhile, honeybees can learn to choose a feeder displaying a d.c. magnetic anomaly with an intensity of just 0.6 per cent of the Earth's magnetic field³. But with an oscillating magnetic anomaly at 60 hertz, threshold intensity required for conditioning is two orders of magnitude higher, an effect consistent with a linear magnetosome chain model (J. Kirschvink, Caltech, Pasadena)⁴. So it looks as if research is closing in on a magnetite-based receptor

in a variety of organisms.

In other systems, such as the eastern red-spotted newt⁵, a different kind of receptor has been implicated, one which shows light-dependent magnetic sensitivity and probably involves specialized



V. Braithwaite

Homing pigeons at home. How do they work out how to get there?

photoreceptors. Light-sensitive magnetoreception is evident in male *Drosophila melanogaster*⁶ too. The receptor can be disrupted by radio frequencies, consistent with theoretical photoreceptor models⁷ relying on interactions between the Earth's magnetic field and electronuclear magnetic moments, which, energetically, are in the radio frequency range (O. Sayeed and J. Phillips, Indiana University). The presence of a fluorescent lamp (a broad-band radio source) in an adjoining room abolished the flies' magnetic orientation, but had no effect when inside a grounded Far-

before birds vanish, and in directions apparently unrelated to the eventual vanishing bearing (C. Spott, Tübingen University). However, large-scale experiments with inexperienced pigeons released at unfamiliar sites 30 km from their home loft in Würzburg, northern Bavaria, reveal a surprising pattern: pigeons usually vanish towards human settlements (J. Kiepenheuer, Max-Planck-Institute, Seewiesen). Topographical influences, for example flying downslope or away from water, have been evident before⁸, but in the Würzburg experiments the 'village effect' is

* Orientation and Navigation: Birds, Humans and Other Animals. 1993 Conference of The Royal Institute of Navigation, Oxford, 15–17 April 1993.

far greater than previously suspected.

As always, the generality of the results may be restricted: these birds had limited training, and no access to olfactory cues on the outward journey. Certainly it is difficult to square the observations with one view expressed at the meeting, that being that release-site biases are probably the result of local inaccuracies in an extrapolated navigational map. This view is partly based on the finding that altering the bird's time-compensated Sun compass (by 'clock-shifting') predictably deflects vanishing bearings at sites with biases just as it does at sites without biases (R. Wiltschko, Frankfurt University). Why should birds be affected by the compass if they are merely being attracted to topographical features like villages? It will be intriguing to learn how clock-shifting works under those conditions producing the village effect.

Perhaps the effect is merely non-navigational: bewildered pigeons head for places offering safety or company. Could it be more? One possibility is that when failing to identify pertinent navigational cues in an unfamiliar area, birds resort to investigating landmarks similar to those in their familiar area. On the edge of the familiar area, such generalization could prove a sensible homing strategy in its own right. So is the village effect visual? Other experiments showed that pigeons in transparent-sided boxes, which were able to 'preview' familiar sites close to (1.4–4.6 km), but out of sight of, the home loft, homed faster than birds in opaque-sided boxes denied this view (V. Braithwaite, Glasgow University). The effect was not apparent at neighbouring unfamiliar sites, suggesting that recognition of familiar visual landmarks has a rather specific role.

Whether the village effect is visual, and to what extent it is even navigational, are sure to become the subject of controversy. But it may well herald a new direction for research, and encourage attempts to understand how pigeons integrate, and decide between, the different strategies they use for orientation in familiar and unfamiliar areas. □

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Magnets without metals

Peter Day

As bars, horseshoes or other more exotic shapes, magnets have been a familiar part of our lives since the days of lodestone. They turn up in nearly every domestic electrical device, and give us the means of recording information on tapes and disks. But what are magnets made of? After lodestone, soft iron in the early days: the Royal Institution has some beautiful examples in its museum which were used by Faraday. In the past 10 years, advances in metallurgy and solid-state physics have given us new magnetic materials such as the rare-earth-cobalt alloys and neodymium iron boride capable of producing very high fields from small volumes (in the words of the trade, they have a high magnetic coercivity). All magnets lose their magnetism above some critical temperature called T_c (the c stands for Curie). 'Permanent' magnets have T_c s far above 300 K, so they are fully magnetized at room temperature. Why, then, should the report on page 147 of this issue¹ that a new magnet has been found with T_c of only 1.48 K be of any interest? As always in materials science, the answer lies in the elemental composition and crystal structure of the new phase.

The crystal structures of the magnets that we are most familiar with consist of continuous lattices of atoms — they are not built from molecules or finite clusters of atoms. Furthermore, such magnets are metals, conductors of electricity. It is also the case that most nonmetallic chemical compounds containing the same elements, such as iron or neodymium, have unpaired electron spins that order antiferromagnetically. That is, the spins on neighbouring metal ions line up in antiparallel fashion, so that there is no net resultant magnetic moment.

Chemists, being of a cussed disposition, have been trying for some years to make molecular-based compounds that are electrically nonconducting but at the same time magnetic. The reason for such an effort is, of course, not just to overturn the prevailing generalizations, but also to create magnets that, unlike metals, are transparent to visible light. Chemists made some progress in the 1970s² in synthesizing materials that changed colour when traversing T_c , but more recent work has concentrated on transition-metal or rare-earth coordination complexes as the building blocks (or bricks, as Olivier Kahn — one of the leading exponents of this approach — has called them). Ingenious molecular design ensures that the unpaired electrons on neighbouring metal ions in the crystal occupy orbitals orthogonal to one another, the quantum-mechanically based recipe for ferro-

magnetism. Compounds of this type have been prepared³ with T_c as high as 30 K. But what about the new T_c of 1.48 K?

In the earliest days of quantum mechanics, Heisenberg⁴ predicted that ferromagnetic long-range order in an infinite lattice would be found only when the lattice contained heavy (that is, metallic) elements. Hence another challenge to the chemist: could one make a molecular ferromagnet that did not contain any metallic elements, and prove Heisenberg wrong? Theorists since Heisenberg, most notably McConnell⁵, have devised mechanisms for ferromagnetic interaction between organic radicals containing only atoms from the first row of the periodic table. These cry out to be tested. Because organic radicals themselves are so chemically reactive, and the compounds difficult to stabilize and characterize, there have been several false alarms on the way to a true organic molecular ferromagnet.

The first such material to pass all the chemical and physical tests was synthesized only in 1991⁶: it goes under the name of *p*-nitrophenyl nitronyl nitroxide and had the less than spectacular T_c of 0.6 K. Still, as Dr Johnson said of the dog walking on its hind legs, the surprise is not that it is done well, but that it is done at all. Several other members of the nitronyl-nitroxide family also show ferromagnetic interactions between neighbouring molecules, but so far without clear evidence of long-range order.

The compound described by Chiarelli *et al.* in this issue¹ also contains nitroxyl groups, on which are located the unpaired electrons. But, whereas in the nitronyl-nitroxide series each molecule contains two N–O moieties connected by a single carbon atom as part of a five-membered ring, in the new compound two N–O groups are deliberately built into the molecule in such a way that the orbitals carrying the unpaired electrons are orthogonal — just the recipe that was followed by the coordination chemists. Thus, even within the molecule, the electron spins on the two N–O groups should align parallel — and they do. More tricky to engineer is the packing of molecules in the crystal, so that the same effect occurs throughout the lattice. However, the susceptibility and magnetization results confirm that that has been achieved. Only one piece of behaviour typical of ferromagnets has not

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been observed, namely hysteresis. Hence (and perhaps not surprisingly), the coercive field is tiny, less than 0.1 oersted.

What of the future? The dog having walked on its hind legs, albeit unsteadily, we can confidently expect T_c s of molecular organic magnets to increase further. Sticking my own neck out, though, I shall be surprised if a magnet containing only carbon, hydrogen, nitrogen and oxygen is ever made with T_c above room temperature. My reasons are twofold: first, the

nature of organic radicals is that they contain only one unpaired electron per repeat unit, and second, the interaction between the orbitals carrying the unpaired electrons takes place across distances equal to the van der Waals intermolecular distance. Both factors militate against high T_c s. Still, the search is fun, and I shall be pleased to be proved wrong. □

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DNA REPAIR

Engagement with transcription

D. Bootsma and J. H. J. Hoeijmakers

ON 18 May 1968 J. E. Cleaver published his discovery that cells of patients suffering from xeroderma pigmentosum carry a DNA-repair deficiency¹. Two papers in this issue^{2,3}, which appear 25 years later almost to the day, are part of a stream of findings which show just how far we have come in understanding repair syndromes since then.

On page 182, Scherly and co-workers² describe the serendipitous cloning of a frog and subsequently a human homologue of the yeast excision repair gene *RAD2*, and demonstrate that this gene is responsible for one of the severe forms of xeroderma pigmentosum (XP-G). On page 185, using an enzymological approach, O'Donovan and Wood³ show that the factor capable of correcting XP-G is identical to the protein missing in rodent cells mutated in the DNA-repair gene *ERCC5*. So the *RAD2*, XP-G correcting protein (XPGC) and

ERCC5 proteins are one and the same. When put into the context of other new developments the implications of this work are far reaching, not least because we can begin to discern the details of a connection between the processes of transcription and nucleotide-excision repair in certain rare disorders.

Nucleotide-excision repair (NER) is a multi-step process that has evolved in all living organisms for the removal of a broad spectrum of DNA lesions. It is central to the prevention of the effects of genotoxic agents, among which is the most ubiquitous environmental threat to DNA, ultraviolet light. The pioneering studies of Hanawalt and his colleagues⁴ have revealed that in most — if not all — organisms there are actually two sub-pathways for NER (see Fig. 1).

Most insights in this field stem from mutants defective in NER. Typically, such mutants display extreme sensitivity to ultraviolet light and to numerous chemicals, as well as elevated levels of induced mutagenesis. The collection of yeast NER mutants currently includes at least 11 members, which make up the *RAD3* epistasis group. Most of the genes involved have been cloned (see ref. 5 for review). Similarly, many mammalian NER mutants have been generated in the laboratory using rodent cell lines. By cell fusion, at least 11 complementation groups have been identified, and by transfection-correction cloning strategies a series of complementing human NER genes has been retrieved (see table).

The phenotypic consequences of an NER defect in humans are apparent from several rare, inborn disorders. Three distinct syndromes are recognized, each characterized by hypersensitivity of the skin to the ultraviolet component of sunlight and by remarkable clinical and genetic heterogeneity^{5,6}. These are XP itself (seven genetic complementation groups, designated XP-A to XP-G); Cockayne's syndrome (two groups, CS-A and CS-B); and PIBIDS, which is a

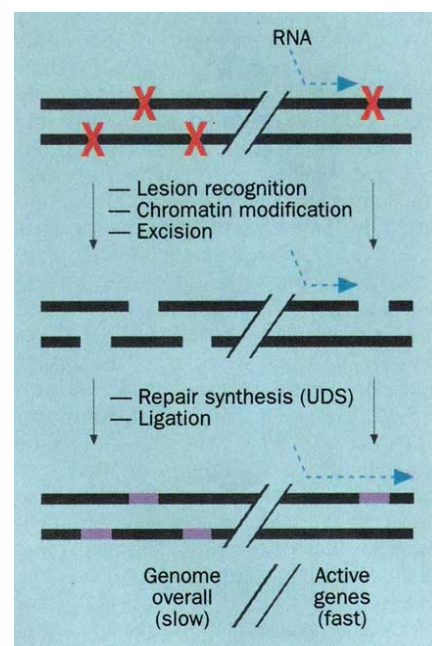


FIG. 1 Steps in nucleotide excision repair. Broadly, the mechanism has five stages: recognition of the lesion; incision of the damaged strand on both sides of the lesion at some distance from it; excision of the injured oligonucleotide; synthesis of new DNA using the intact complementary strand as template; and ligation. Two NER subpathways can be discerned. One is the rapid and efficient repair of the transcribed strand of active genes (transcription-coupled repair). The other is the slower and less efficient repair of the bulk DNA, including the non-transcribed strand (genome overall repair).

peculiar photosensitive form of the brittle-hair disease trichothiodystrophy (at least two groups, TTD1BR and one equivalent to XP-D).

As well as sensitivity to sunlight, other manifestations of XP include pigmentation abnormalities and a more than 2,000-fold increased frequency of skin cancer, often accompanied by progressive neurological degeneration. In the case of CS, neurological dysfunction is associated with dysmyelination of neurons, which could be due to poor expression of one of the myelin proteins, and this disease is also characterized by poor physical and sexual development. Patients with PIBIDS manifest all the CS symptoms and, curiously, two hallmarks of trichothiodystrophy: ichthyosis, and brittle hair and nails (the brittleness being due to a reduced content of a cysteine-rich matrix protein). Intriguingly, individuals with CS and PIBIDS do not seem to be unduly prone to cancer. This remarkable clinical heterogeneity associated with NER impairment is even more perplexing because defects in one gene may give rise to symptoms of XP, combined XP/CS or PIBIDS. Notably, this holds for XP-B, XP-D and XP-G⁷, and implies that all the syndromes are

EUKARYOTIC NUCLEOTIDE-EXCISION-REPAIR GENES AND MUTANTS

Human mutant (and gene)*	Rodent mutant†	Yeast gene
XP-A (<i>XPAC</i>)	?	<i>RAD14</i>
XP-B (<i>XPBC</i>)	<i>ERCC3</i>	<i>RAD25 (SSL2)</i>
XP-C (<i>XPCC</i>)	?	<i>RAD4?‡</i>
XP-D (<i>XPDC</i>)	<i>ERCC2</i>	<i>RAD3</i>
XP-E	?	?
XP-F	?	?
XP-G (<i>XPGC</i>)	<i>ERCC5</i>	<i>RAD2</i>
CS-A	?	?
CS-B (<i>CSBC</i>)	<i>ERCC6</i>	?
TTD1BR	?	?
?	<i>ERCC1</i>	<i>RAD10</i>
?	?	<i>RAD1</i>
?	?	<i>RAD7</i>
?	?	<i>RAD16</i>

* *XPAC* (and so on) is the term for the gene encoding a protein that corrects XP-A.

† Rodent complementation groups are numbered. The human genes correcting the defect of rodent mutants are designated *ERCC* (for excision repair cross complementing) genes, the number referring to the number of the corrected group.

‡ Level and type of homology not conclusive.

manifestations of a much broader clinical entity.

Molecular genetic analysis has revealed striking homology between all eukaryotic NER genes. Indeed, the entire NER system seems to be highly conserved from yeast to man, the gene products in yeast, rodents and humans fitting as pieces of the same puzzle (see table). The cloning of the *XPGC* gene by Scherly *et al.*² and the *in vitro* complementation studies of O'Donovan and Wood³ fill in two of the remaining holes: *XPGC* is the human homologue of *RAD2*, and *ERCC5* is probably *XPGC*. The primary amino-acid sequence does not reveal any secrets, however, so this protein's role remains enigmatic.

Putting the clinical and molecular observations into a broader perspective, a puzzling question is how such features as neurodysmyelination, retarded development and brittle hair can be interpreted in terms of a repair defect. A first hint that other aspects of cell metabolism are also affected in some of these mutants came from the yeast homologues of the XPBC and XPDC proteins. The corresponding genes appeared to have another function in an unknown process essential for yeast viability⁸⁻¹¹, and the nature of that process was uncovered only a few weeks ago in a completely unrelated line of research.

Egly's group in Strasbourg has a long-standing interest in the molecular mechanism of transcription initiation of most of our genes (transcribed by RNA polymerase II). While searching for a transcription factor they stumbled upon a repair gene, and last month reported the identification of the repair protein XPBC/ERCC3 as part of the basal transcription factor TFIIF¹². In TFIIF, ERCC3 seems to fulfil a DNA-melting function as an obligatory late step in the complex process of transcription initiation. The DNA-unwinding function was already anticipated on the basis of the primary amino-acid sequence¹³.

This rediscovery of the same protein in a different context points to several conclusions. First, the CS features of

neurodysmyelination and retarded development typical of XP-B, which are reminiscent more of a defect in expression than in NER, may reflect a subtle impairment of general transcription. This proposition also provides an explanation for the curious phenotype of the XP-B equivalent in *Drosophila*, 'haywire', which has defects in the central nervous system and in its sexual

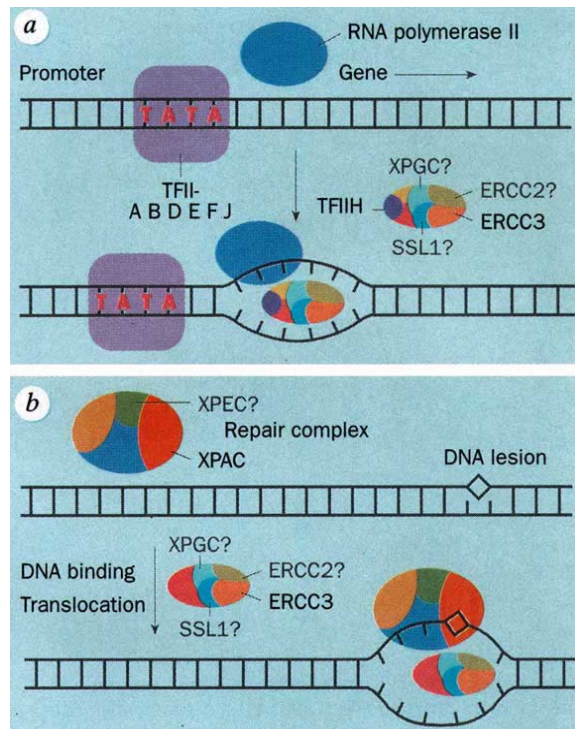


FIG 2. Models for the role of ERCC3 in transcription and nucleotide excision repair. *a*, Possible function of ERCC3 and the TFIIF transcription factor in transcription initiation by RNA polymerase II. Various factors including TFIIF, B, D, E, F and J assemble on the promoter (upper part). The local melting activity of the ERCC3 helicase in the TFIIF factor may be required for loading the RNA polymerase onto the template and/or for translocation of the complex along the DNA during the elongation reaction (lower part). *b*, Possible function of ERCC3 in NER. The local DNA-melting activity of ERCC3, perhaps in the context of a TFIIF-like factor, may be required for assembling a DNA-damage-recognition complex onto the DNA, for translocation along the helix, or for both. Alternatively, or in addition, ERCC3 may be involved in inducing a specific, locally denatured conformation in the DNA at the site of the lesion as a prerequisite for the binding and action of the NER-incision complex.

development¹⁴. The same may hold for the symptoms of CS and brittle-hair trichothiodystrophy in XP-D, which in many respects are very similar to XP-B. As such these complex disorders may define a new class of transcription syndromes that may also occur in the absence of an associated repair deficiency (for instance trichothiodystrophy without photosensitivity).

Second, the new findings imply that a subset of proteins is involved in two quite different aspects of DNA metabolism, initiation of transcription and NER.

A model for a similar function of the ERCC3 protein in both processes is that it carries out DNA unwinding coupled to translocation of the transcription complex (Fig. 2*a*) and the repair complex (Fig. 2*b*) along the helix. Alternatively (or in addition) it may be involved in inducing a specific DNA conformation for loading RNA polymerase or the NER-incision complex onto the DNA. Because XPBC/ERCC3 is part of the multi-protein TFIIF factor, it would not be a complete surprise if other repair products turn out to be hidden in this or a similar complex. One candidate is XPDC/ERCC2/RAD3, because this protein is also a DNA helicase and the corresponding mutants resemble XPBC/ERCC3/RAD25 mutants in many respects^{5,6,13}. Another is the uncloned human homologue of *SSL1* that seems to interact with *RAD25* (*SSL2*)^{10,15}. XP-G, like XP-B and XP-D, belongs to the exceptional class of groups harbouring patients with combined XP/CS⁷, so it may turn out that XPGC is also involved.

The relationship between transcription and repair, as revealed by Hanawalt and colleagues, therefore now reaches the stage of an official engagement. Indeed, it fits nicely into the emerging pattern that the eukaryotic NER system recruits many of its factors from proteins already involved in other basic aspects of DNA metabolism such as replication¹⁶ and recombination¹⁷. Finally, the concept of 'transcription syndromes' in the human population is remarkable in itself. Not only does it show that extremely rare disorders can still be very revealing, but it will open a new chapter in human genetics as well. □

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The nucleon in a spin

A. D. Martin

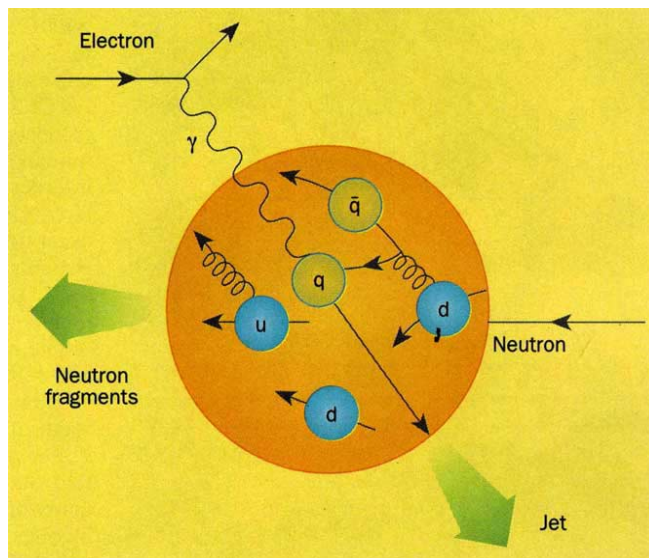
THE proton, composed of quarks and gluons, should carry its constituents' signature. But measurements of the proton's spin structure at CERN five years ago found a surprising result: very little of the proton's spin seemed to be carried by the quarks, leaving it to the gluons and/or orbital motion. A burst of theoretical activity ensued and planning began for two independent experiments to make measurements of the spin structure of the neutron, the twin particle of the proton. Indeed, apart from their electric charge, the neutron and proton are so similar that they are given a single name: the nucleon. Both these neutron experiments, one at CERN, the other at Stanford, have reported results within the past few weeks.

Polarization experiments involving neutrons present a severe technical challenge. In the new CERN experiment¹, performed by the Spin Muon Collaboration (SMC), the target contains frozen, polarized deuterons (each comprising a proton and a neutron). The other experimenters², at the Stanford accelerator in California, use a gas target of polarized helium-3 nuclei, each composed of two protons and a neutron. The advantage of this arrangement is that the two protons almost always have opposite spins, so that their contributions cancel and the whole nucleus acts essentially like a polarized neutron for the purposes of the experiment.

In each experiment, so-called deep inelastic scattering (the high-energy equivalent of Rutherford's atomic structure experiments) is used to probe the interior of the neutron. Polarized electrons are scattered off the quarks in the Stanford target and detected, whereas polarized muons are used in the CERN experiment. In fact the CERN target is divided into two halves which are longitudinally polarized in opposite directions so data can be recorded simultaneously for scattering with the muon and neutron spins both parallel and antiparallel.

The figure shows a schematic sketch of deep inelastic electron-neutron scattering in which possible internal structure of the neutron is exposed. The neutron contains three valence quarks, one up and two down quarks: udd. These may radiate gluons, which are the mediating particles

responsible for the quantum-chromodynamic (QCD) interaction between quarks (which, in turn, is the basis of the strong nuclear force). These gluons may transform into quark-antiquark ($q\bar{q}$) pairs. In principle, the neutron (or the proton) can contain any number of $q\bar{q}$ pairs. At the energies of the experiments, they could be $u\bar{u}$, $d\bar{d}$ or $s\bar{s}$ pairs where s is



Deep inelastic scattering viewed from a frame in which the electron and neutron are incident from the left and right respectively. The partonic structure of the neutron is shown: the valence (ddu) and sea ($q\bar{q}$) quarks, and the gluons (curly lines). The spin of the neutron has components from the spin- $\frac{1}{2}$ quarks, spin-1 gluons and their relative orbital angular momenta. When probed at high energies the partons (the quarks and gluons) act as if they are almost free point particles inside the neutron, but are subject to the strong confining QCD or 'colour' force if they try to escape. Therefore the parton that is struck by the photon (a sea quark in this example) actually materializes as a 'colourless' jet of particles (hadrons). An identical picture describes electron-proton deep inelastic scattering except that the proton has uud valence quarks.

the strange quark. The $q\bar{q}$ pairs are known as the sea quarks. Deep inelastic electron-neutron scattering takes place by the exchange of a photon (γ). The more energetic the interaction, the shorter the wavelength of the photon and the more detailed the quark structure of the neutron that is probed. One of the beauties of the QCD-parton model is that the scattering is incoherent; we simply add the probabilities of the scattering from the various valence or sea quarks. (There is no scattering from the gluons, because they do not carry electric charge.)

Taking into account the various particle spins, the neutron (probed as in the figure) is described in terms of four structure functions, each of which can be expressed as a particular sum of the probabilities of scattering from the various component quarks carrying a fraction x of the neut-

ron's momentum. The new experiments measure the structure function known as $g_1^n(x)$, which is essentially the difference of probabilities of scattering from quarks with spin parallel and antiparallel to the electron spin (which in turn is along the direction of motion). The quantity of particular theoretical interest is the integral I_n of $g_1^n(x)$ over all possible momentum fractions, that is $0 \leq x \leq 1$. An analogous integral I_p for muon-proton deep inelastic scattering was determined in the 1988 CERN polarization experiment^{3,4} by EMC (for European Muon Collaboration). That experiment found, after extrapolation over the unmeasured regions of x , that $I_p = 0.126 \pm 0.018$.

There is a fundamental sum rule, originally derived by Bjorken⁵ from the algebra of the electromagnetic and weak currents of the different types of quarks, but later shown to be a rigorous prediction of QCD, which, at present interaction energies, gives $I_p - I_n = 0.191 \pm 0.004$. Clearly polarization measurements on both neutrons and protons are needed to test this important prediction. However, by making an additional assumption that the strange-quark sea (the $s\bar{s}$ pairs) within the proton (and neutron) is unpolarized, Ellis and Jaffe⁶ deduced sum rules for I_p and I_n separately. These predict $I_p = 0.175 \pm 0.010$ and $I_n = -0.016 \pm 0.010$.

The discrepancy between the prediction for the proton and the EMC experimental value was the cause of the excitement in 1988. Clearly there was a need for a determination of I_n using polarized neutrons. Now, five years on, two experiments have simultaneously reported measurements of this.

The SMC polarization experiment¹ on deuterons finds $I_n + I_p = 0.049 \pm 0.054$, which, on combining with I_p of EMC, yields $I_n = -0.08 \pm 0.06$ and a difference compatible with (though not, as yet, a stringent test of) the Bjorken sum rule. With reasonable assumptions, the SMC experimenters deduce that the quarks carry, in total, only 6 ± 25 per cent of the neutron's spin, consistent with the surprise conclusion of the 1988 proton experiment. SMC is an ongoing experiment and it will be interesting to see if this result survives the increase in their precision.

On the other hand the Stanford experiment², performed by the E142 collaboration at a rather low interaction energy, finds $I_n = -0.022 \pm 0.011$, in good agreement with the Ellis-Jaffe sum rule. But, if combined with I_p of EMC, it is

Immunological agnosia

Paul Travers

ALTHOUGH the immune system is armed cap-à-pie with all manner of offensive weapons, and is ready to respond to the slightest insult, it fails to respond to the myriad self-antigens presented throughout the body. How so? The issues are many and convoluted, but on page 156 of this issue¹ Sloan-Lancaster *et al.* describe a hitherto unknown mechanism by which T cells can be inactivated.

Two distinct phenomena underlie the nonresponsiveness of the immune system to self-antigens. The first, central or thymic tolerance, results from the death of developing T cells able to recognize self-antigens expressed within the thymus², the specialized microenvironment in which T-cell development takes place. The second, peripheral tolerance, occurs after T cells have left the thymus and accounts for the failure of the immune system to respond to self-antigens that it cannot have encountered in the thymus.

The mechanisms underlying thymic tolerance are poorly understood, because the development of T cells in the thymus contains a paradox — to survive, a T cell must express a receptor capable of recognizing a self-molecule of the major histocompatibility complex (presumably one occupied by a self-antigen), a phenomenon known as positive selection; yet T cells that do recognize self-antigen-MHC complexes are induced to die (negative selection)^{3,4}. To escape this paradox, it is generally considered that it is the strength of the interaction between the T-cell receptor and MHC plus peptide that is decisive. T cells are initially programmed to die; those whose receptor has a high enough affinity for self-MHC plus some undefined peptide ligand(s) receive a signal that enables them to survive; and those which encounter a self-antigen for which the receptor has high affinity are induced to die.

The mechanisms of peripheral tolerance are better understood (or at least we think they are). Under normal circumstances, when a resting T cell comes into contact with an antigen-presenting cell displaying both the appropriate ligand-MHC complex and the costimulatory molecule, B7/BB1 (ref. 5), it proliferates and differentiates into an effector T cell. Both the antigen-specific signal delivered through the T-cell receptor and the costimulatory signal, delivered through CD28, are required⁶. Ligation of the T-cell receptor in the absence of the costimulatory signal leads to the T cell becoming nonresponsive, a condition known as anergy⁷⁻⁹. It is now relatively clear that the function of the second signal is to induce the secretion of interleukin-2 by the T cell and

so to drive proliferation⁹. Most cells and tissues do not express B7/BB1 and thus cannot activate resting T cells; they will, moreover, render unresponsive any T cells bearing receptors capable of recognizing self antigens.

Less attention has been paid to the involvement of the T-cell receptor in establishing peripheral tolerance, it being seen as tautologous that a T cell which cannot recognize antigen cannot respond. The relationship between the affinity of the receptor and the ability to generate a response has been seen as a simple one, with a threshold of affinity above which the T-cell receptor would bind and stimulate a response and below which there would be no binding and no response.

The data of Sloan-Lancaster *et al.*¹ now suggest that the affinity of the interaction between T-cell receptor and MHC-peptide plays a more important role, and that there is no simple threshold but a fuzzy no-man's-land. Within this no-man's-land, the affinity of interaction between the receptor and the MHC-peptide ligand is such that signalling by the receptor can occur (the receptor can see the ligand) but signalling is insufficient to induce the full, proliferative response. Instead, the T cell becomes nonresponsive, that is, anergic.

Sloan-Lancaster *et al.* have shown, in an *in vitro* system where T-cell clones are stimulated by defined peptide antigens presented by live, functional antigen-presenting cells, that modifying residues on the peptide which contact the T-cell receptor¹⁰ leads to a state of partial activation of the T cell and the subsequent unresponsiveness of the T cell to stimulation with the wild-type, fully stimulatory peptide. Two substitutions were made in a murine haemoglobin peptide. When presented with one of these analogue peptides (Ser-70), the cloned T cells respond by increasing in size and upregulating both the interleukin-2 receptor and the integrin, LFA-1, but do not proliferate or secrete cytokines. More importantly, once they have been stimulated with the analogue peptide, the T cells are unresponsive to stimulation with the wild-type peptide; they have become anergic.

The second analogue peptide (Gln-72) also fails to induce proliferation by the T-cell clone but does not induce anergy. In this case the analogue peptide appears to have too low an affinity to stimulate the T cells at all (consistent with the role of the wild-type residue, Asn-72, as the primary T-cell contact residue¹⁰). So three classes of peptide affinity can be defined — good (the wild-type ligand), bad (the lowest affinity, nonstimulatory ligands) and ugly

Tests of quark sum rules

$$I_p - I_n$$

Bjorken rule	0.191 ± 0.004
SMC + EMC	0.20 ± 0.06
E142 + EMC	0.148 ± 0.022

$$I_n$$

Ellis-Jaffe rule	-0.016 ± 0.010
SMC + EMC	-0.08 ± 0.06
E142	-0.022 ± 0.011

$$I_p$$

Ellis-Jaffe rule	0.175 ± 0.010
EMC	0.126 ± 0.018

EMC, SMC and E142 are the three polarized lepton-nucleon deep-inelastic-scattering experiments described in the text. The sum-rule predictions depend weakly on the experimental interaction energy.

about two standard deviations below the more fundamental Bjorken sum rule. The authors deduce that, in total, the quarks carry 57 ± 11 per cent of the neutron's spin; a more precise determination than that of SMC and more in line with expectations.

From the various results, gathered together in the table, it is apparent that the present situation is inconclusive. Although the two determinations of I_n are compatible, the SMC errors are large. The collaboration has more data to analyse and so will be able to increase the precision. Will the improved measurement remain compatible with the more accurate E142 result? If not, then there may be doubts as to whether the interaction energy of the E142 experiment is sufficiently large for perturbative QCD to provide an accurate theoretical framework for their data. Clearly an independent and more precise measurement of I_p of the proton is essential. SMC will do this. Also a new dedicated experiment to measure both integrals using a gas jet of polarized nucleons as a target is being planned at the accelerator laboratory, HERA, in Hamburg. Will the Bjorken sum rule survive the more detailed scrutiny? This is the first of many fundamental questions of the spin structure of the nucleon which will continue to intrigue particle physicists for some time to come.

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Heavenly music

A VENTRILOQUIST makes his voice seem to come from a distant source. Daedalus is now inventing a true, large-scale ventriloquism. It exploits those microwave frequencies that are feebly absorbed by water vapour in the air. His novel phased-array radar directs such an absorptively lossy beam upwards, and sweeps it angularly through the atmosphere.

The absorption of the beam heats and expands the air through which it travels. As the beam sweeps sideways, the expansion moves too. At some height in the atmosphere, determined by the angular sweep rate, the beam sweeps laterally at exactly the local speed of sound. The accompanying moving expansion launches a horizontal sound wave into the atmosphere at this precise height. If the sideways sweep is accelerated, the sonic height moves steadily downwards, tilting the sound beam down towards the ground. So by sweeping a spatially audio-modulated beam sideways, while updating it continuously at the trailing edge, Daedalus can create a disembodied voice which speaks from the sky.

The primary interest is military. The US army attacked General Noriega with a non-stop barrage of deafening rock music; the FBI used similar tactics in the Waco siege. The ghastly sonic barrage maddened its own side as well. Ventriloquial radar solves this problem: it makes no noise itself. Aimed into the sky, the silent beam can target any site within many kilometres, and stun it with kilowatts of accurately directed rock music. With enough computer power, it could be trained on a moving target such as an aircraft, following it around the sky till its distracted crew abandoned their mission. They might even conclude that the deafening, sourceless voice was inside their own heads, and that they were succumbing to schizophrenia.

Civilian uses would soon follow. As a free-air public-address system, ventriloquial radar could beam a celestial sports commentary or a pop sound-track precisely down onto a stadium, while leaving the surrounding area in perfect peace. It could also launch horizontal signals at high altitudes for scientific purposes. Like the ocean, the atmosphere may have confining layers within which sound travels horizontally for vast distances. The transit time, frequency response and distortion imposed on such signals would neatly reveal a vast amount about the air through which they had travelled.

David Jones

(the intermediate affinity, tolerogenic ligands).

In that the T cells no longer respond to a normally stimulatory peptide antigen, they have become anergic. But this state differs from that induced by T-cell stimulation in the absence of a costimulator. In the latter case, T-cell responses can be restored by adding an exogenous source of costimulation, such as allogenic spleen cells or anti-CD28 antibody⁶, whereas, when suboptimal peptides are used, exogenous costimulation does not restore the response. Clearly then, these two types of anergic cells differ, which must result from the difference in signals transduced by the T-cell receptor upon engagement of ligands of greater or lesser affinity.

How then are we to understand the nature of the signals given by the analogue peptides that result in the induction of anergy? Simply reducing the number of T-cell receptors engaged cannot account for the observations, because limiting amounts of the fully stimulatory peptides would have the same effect. I can envisage two possible mechanisms that could account for a differential signal. One is that the affinity of the receptor-MHC-peptide complex is now sufficiently low that the complex is short-lived; the other is that conformational change in the T-cell receptor may be required for efficient signal transduction, perhaps to allow interactions with the coreceptor, CD4 or CD8, and that the binding of the analogue peptides does not induce the change in conformation.

In either case it is possible, with appropriate handwaving, to suggest that the signal transduced by the T-cell receptor complex may be different to that transduced when it is fully engaged. Exactly how these signals differ is less clear. Production of inositol phosphates in the T cells, an indicator of the activation of a signalling pathway required to induce the influx of Ca^{2+} , is not increased on stimulation with the suboptimal analogues (although it can be argued that this assay is not sensitive enough to pick up the small amount of inositol-1,4,5- P_3 that would be required to induce a Ca^{2+} flux¹¹). A measurement of intracellular Ca^{2+} itself would have been useful in dissecting the signalling events after stimulation with suboptimal peptide analogues, as cyclosporin A, which inhibits the Ca^{2+} -dependent signal transduced by the T-cell receptor, inhibits the establishment of the anergic state. This observation, which at face value is inconsistent with the failure to see increased levels of inositol phosphates in the T cells, may imply either that calcineurin, which is the target for cyclosporin A inhibition¹², is constitutively activated in the T-cell clones used by Sloan-Lancaster *et al.* and does not require a Ca^{2+} signal, or that the partial

signal given by the suboptimal peptide analogues results in the production of enough inositol-1,4,5- P_3 to initiate a Ca^{2+} flux but too little to be detected by the assay used.

Whatever the mechanisms, the ability of T cells to be rendered anergic by suboptimal ligands poses both an opportunity and a problem. The opportunity is that potentially disadvantageous immune responses to defined epitopes, say in allergic responses, may be regulated by administering appropriate analogue peptides to anergize potentially responsive T cells. The problem is in understanding how any of our T cells manage to make responses at all. The nature of positive selection of T cells during thymic maturation supposes that there are low-affinity interactions, presumably mediated by self-MHC molecules complexed with some or other self-peptides, which signal to thymocytes that they express a functional receptor and which rescue the thymocytes from programmed cell death. All T cells therefore have a specificity which can be described as 'slightly different from self'. So are all of our T cells rendered anergic by self-antigens?

The effect of suboptimal peptide ligands shows that for T cells, as for humans, the process of recognition is a complex one, being composed of both seeing and perceiving. A fascinating insight into human perception comes from the study of agnosia, a condition in which the patient can see an object, and indeed describe it in detail (a hand-shaped sack with five pouches), while not being able to identify it (a glove). The anergy induced by suboptimal peptides is similar in that the T cells can 'see' the peptide (the receptor can bind the MHC-peptide complex) but not recognize it (the T cell does not make an effective response). Because failure of recognition is associated with partially stimulatory analogues rather than the cognate antigen, perhaps we have the case of the T cell who mistook his mother-in-law for a hat¹³. □

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Aluminium – Alzheimer's link?

SIR — Landsberg *et al.*¹ claim that “the most direct evidence for the link between aluminium and Alzheimer's disease stems from reports of granular aluminosilicates found in neuritic plaque cores”. This hypothesis is not supported by evidence. Terry *et al.*, for example, have found no correlation between numbers of plaques and severity of dementia in Alzheimer's patients; a weak correlation with neurofibrillary tangle density; and a high correlation with synaptophysin-containing neurites². This raises the question of whether aluminium associated with plaques would be relevant to the clinically important dementia of Alzheimer's. If aluminium does play a role, it is important to know its localization to examine its pathological significance.

Landsberg *et al.* state that their novel instrumentation (PIXE) is highly sensitive. Having failed to demonstrate the presence of aluminium in neuritic plaque cores in unstained sections, they conclude that demonstration of aluminium by previous research using other methodology was probably due to contamination. Yet Landsberg *et al.* did not present positive controls in the form of cellular matrix-bound aluminium. Their specimen, stated to be from the brain of a patient with Alzheimer's, would have contained neuritic plaques interspersed with tangle-bearing neurons. If Landsberg *et al.* had looked at these tangles or neuronal nuclei and showed the presence of matrix-bound aluminium in neurofibrillary tangles or in nuclei, significant knowledge would have been gained regarding the use of their novel technique to answer questions of histological aluminium compartmentalization. This would have been of particular importance because the presence of aluminium in neurofibrillary tangles in unstained sections using different instrumentation and procedures has clearly been demonstrated (see the recent Scientific Correspondence by Good and Perl³). Further, a study by Lovell *et al.*⁴ clearly shows aluminium accumulation in nuclei and neurofibrillary tangles of Alzheimer's brains using laser microprobe analysis. We have shown, using electrothermal atomic absorption spectrophotometry, that nucleosomal fractions from Alzheimer's brains contain up to nine times as much aluminium as controls⁵.

Having failed to demonstrate aluminium in neuritic plaques while being able to detect contaminating aluminosilicate particles, Landsberg *et al.* state “previous evidence that aluminium is involved in the aetiology of Alzheimer's disease should be reviewed”. With this

conclusion the authors disregard or challenge all previous work relating aluminium and aluminium-induced toxic effects on the central nervous system in people with Alzheimer's⁶. Contrary to the opinion of Landsberg *et al.*, which was widely quoted by the popular press, we believe that evidence suggesting a link between aluminium and the pathogenic process in Alzheimer's disease cannot be dismissed.

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Illusion and view stability

SIR — Carman and Welch¹ argue that illusory contours and surfaces retain their shape over changes in position and orientation relative to the observer (view stability). We would like to provide an example of illusory surfaces that do not exhibit three-dimensional view stability. The inducing pattern was devised by W. Ehrenstein² and is shown in Fig. 1a. When this pattern is viewed at arm's length straight on, the illusory bright patches observed are round and delineated by circular illusory contours. When the pattern is viewed at a slanted angle as described in the figure legend, the illusory patches become deformed and assume oval or squashed shapes.

The illusory surfaces used by Carman and Welch are induced by solid luminance edges while those shown in Fig. 1 are induced by the ends of lines. It has been proposed that these two types of illusory surfaces are induced by different mechanisms³, and they may have different neurophysiological correlates^{4,5}. Moreover, Watanabe and Cavanagh⁶ showed that, generally, illusory surfaces induced by solid luminance edges resist depth capture while illusory surfaces induced by line ends do not.

Note that, under the slanted viewing conditions, the black lattice inducing the illusory patches and the page on which it is printed are perceived as shape-invariant. Nevertheless, there is no view stability for the illusory patches. This finding may indicate that the process of morphopsis¹ is based on local informa-

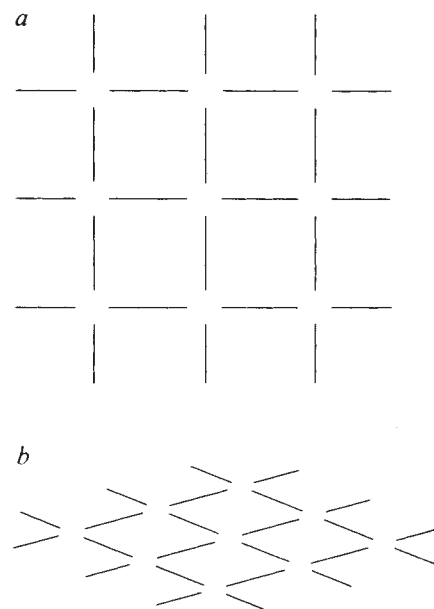


FIG. 1a, Ehrenstein's original pattern². Bright illusory patches are seen in each gap. When this pattern is viewed at arm's length straight on (visual axis perpendicular to the page), these patches usually appear round and are delineated by a circular illusory contour. When the page is now tilted so that the Ehrenstein pattern is seen at a slanted angle, the illusory patches are no longer round but assume oval or squashed shapes, depending on the degree of tilt. This deformation is more pronounced if the slanted pattern is viewed from the side (along one of the diagonals) (b). The deformed illusory patches in a pattern presented this way have a shape similar to patches induced by a correspondingly pre-deformed pattern that is viewed straight on (b). This indicates that the position of the observer, relative to the inducing pattern, has little or no influence on the form of the illusory patches under these conditions, and there is no three-dimensional view stability.

tion restricted to the immediate surroundings of the illusory surface, that is, to the line ends, rather than on more global features of the Ehrenstein lattice. The binocular disparity signals provided by the line ends are probably too weak⁶ to generate view stability.

Although our observations do not necessarily contradict the general concept of view stability proposed by Carman and Welch, they do provide an exception which awaits an explanation.

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CARMAN AND WELCH REPLY — Redies and Watanabe claim a lack of view stability for the Ehrenstein grid². But we found that only 4 out of 10 observers reported perceiving the distortion of the illusory circles into ovals when Fig. 1a is slanted and tilted in the manner suggested by Fig. 1b. We note that Fig. 1b

ate an infinite variety of boundary contours⁷, we expect that two-dimensional boundary contours would deform, while their underlying three-dimensional shape would be preserved under conditions of inconsistent or ambiguous cues. Such observations are entirely consistent with our notion of view

High Resolution Radiometers) of each of the two satellites scan over each surface area twice a day, once during the day and once at night, with a nominal time displacement of around 6 hours. Over a 2-week period in March 1988, 26 Gambian foresters reported all continuing and burned-out savanna fires which had occurred at the NOAA overpass times along pre-determined routes. The foresters drove along the routes twice a day, once just before sunset and once just after dawn. They reported a total of 115 fires which showed a strong diurnal cycle. The minimum fire frequency occurred at the 2.30 a.m. local time overpass (NOAA-9). At the 8.30 a.m. pass (NOAA-10) the fire frequency was 6.3 times higher; at the 2.30 p.m. pass (NOAA-9) 8.5 times higher; and at the 8.30 p.m. pass (NOAA-10) it was 2.8 times higher.

Further, I have analysed eight pairs of consecutive NOAA-10 evening and NOAA-11 early-morning images of Guinea-Bissau, the Gambia, Senegal and parts of Mauritania, Mali and Guinea from November 1989 to May 1990 using a physically based bispectral interpretation model³. The model uses AVHRR thermal night-time data in the 3.55–3.92- μm and 10.2–11.2- μm bands and has been tailored for night-time detection of savanna fires. These pairs enabled analysis of the relative frequencies of fires at around 10 p.m. and 3 a.m. local time. The relative fire frequencies between the two night-time passes were very similar to those obtained in the field; namely 2.7 to 1 for the late-evening pass compared to the early-morning pass.

Even though most savanna fires in West Africa are not controlled, this does not necessarily imply that night-time fire frequencies are close to day-time frequencies, as suggested by Cahoon *et al.* At least in the region covered by my study, the weather conditions counteract biomass burning at night because of increased air humidity and associated dew deposition on the vegetation, reduced wind activity and lowered air temperature. Under low wind speeds the normal duration of savanna fires, most of which are started, intentionally or accidentally, by the rural population, is determined by the time before the fire front is trapped by natural or artificial barriers. This usually happens within 12 hours, or before dawn the next day.

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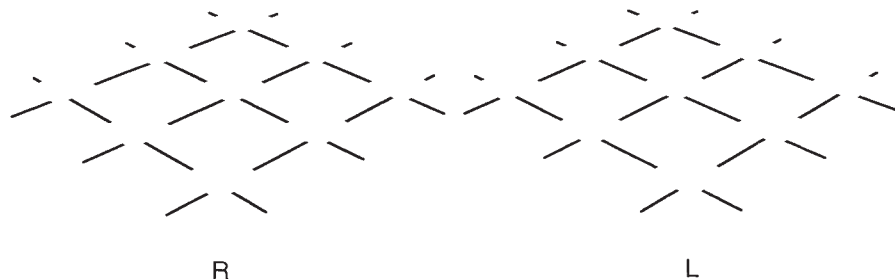


FIG. 2 View stability of the three-dimensional Ehrenstein grid. Hold figure as close as possible while viewing L with the left eye and R with the right eye. This approximates the natural viewing of a three-dimensional Ehrenstein grid whose intersections were occluded by small planar squares having no contrast with the surround. Observers report perceiving planar illusory squares slightly raised above the intersections of the grid, and do not perceive the distortions seen under conditions of inconsistent perspective and stereo cues. (Methods described in ref. 1.)

presents monocular perspective cues consistent with a grid tilted away from the plane of the page, but binocular disparity cues consistent with a grid coplanar with the page.

That such inconsistent combinations of cues can result in perceptual ambiguity or distortion does not constitute evidence that illusory surfaces are induced by separate mechanisms for different figural cues. Indeed, when we simulate natural viewing of a three-dimensional Ehrenstein grid with consistent perspective and disparity cues, the illusory surface patches which are induced at the grid intersections are perceived as squares over a wide range of views (see Fig. 2). Furthermore, we find that we can induce various three-dimensional illusory contours and surfaces using random dots or line segments as well as luminance cues, and that perception of these illusory forms is characterized by the same cue invariance, view stability and morphic generality as reported in our original study¹.

Those who do perceive this illusion report only the deformation of the boundary contour, while the underlying shape of the illusory surfaces remains planar over change in view. Because any three-dimensional form can gener-

stability, which is properly understood as invariance of the perceived intrinsic three-dimensional shape or curvature of objects, but not necessarily of their apparent boundary contours, under changes in orientation with respect to the observer^{1,8}.

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Diurnal cycles in savanna fires

SIR — Cahoon *et al.*¹ reported the temporal and spatial distribution of savanna fires in continental Africa derived from interpretation of hard-copy satellite images of the Defense Meteorological Satellite Program (DMSP) Block 5 satellites. In their study they examined images measured in the 0.4–1.1 μm range from the 1986 and 1987 fire seasons. All images were acquired at local midnight. They found, against their expectations, that “most fires are left to burn uncontrolled, so that there is no strong diurnal cycle in the fire frequency”.

I found contrary results using field observation and thermal satellite images². The field work was designed to provide fire frequencies in the Gambia at the four daily satellite overpasses of two orbiting NOAA (National Oceanic and Atmospheric Administration) satellites. The AVHRR (Advanced Very

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Appliances of science

Jack Meadows

Before the Computer: IBM, NCR, Burroughs, and Remington Rand and the Industry They Created, 1865–1956. By James W. Cortada. Princeton University Press: 1993. Pp. 344. \$55, £39.50.

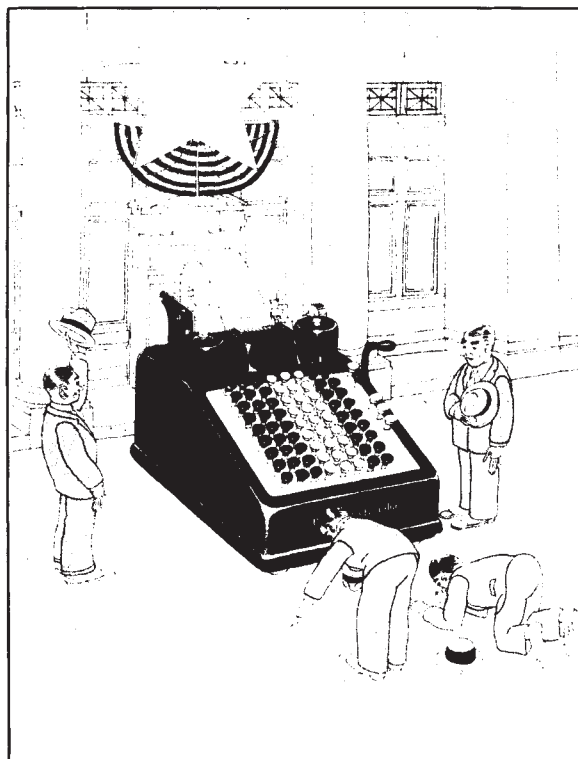
Our reputation sparkles like a gem!
We've fought our way through — and new
Fields we're sure to conquer too
For the EVER ONWARD IBM.

THIS anthem was written in the 1930s, long before IBM (International Business Machines) became known for its electronic computers. It reflects the early importance of the company to the industry examined here — the provision of office appliances for mechanized data handling. This automation of 'white collar' work, Cortada suggests, was triggered by a series of nineteenth-century innovations — the typewriter, the cash register and the adding machine. The industry based on these appliances was already well established by the beginning of the First World War; and its leading companies subsequently spearheaded the introduction of the electronic computer.

The growth of the industry was obviously based on technological change; but, equally, it depended on a changing marketplace. During the nineteenth century, the manufacturing and service sectors in Europe and North America grew rapidly, in the process generating a vast amount of data. Office workers were recruited to handle this flow of data, but, as it escalated, manual methods often proved inadequate. And so, logically, came mechanization. The need for this was made clear when Herman Hollerith mechanized the handling of the data gathered for the US census of 1890. With his machines, an analysis that manually would have taken ten years was compressed into two years, and the cost reduced by \$5 million.

The success of Hollerith's machines was due to two factors — good marketing and good technology. Cortada sees these as the basic determinants of success in the burgeoning office appliance industry. Sales representatives were treated as professionals by employers earlier, and to a greater extent, than in most other industries. Companies such as NCR (National Cash Register Company) and, subsequently, IBM pioneered systematic sales techniques — quota targets, exclusive terri-

tories and so on. The basic reason for such professionalism was the nature of the products being sold. They were expensive, and purchasers expected to talk to knowledgeable sales staff who would also provide after-sales service. So sales personnel needed to have a similar background and lifestyle to



Cartoon from *Burroughs Journal*, 1926. The caption reads: "Our Deepest Gratitude, Little One — You Brought Us Here".

the business managers with whom they dealt. Thus, in later years, sales staff were increasingly college graduates.

We are accustomed to think of much of the computer industry as technology-driven. This was less true of the early data-handling industry. Changes occurred, but typically in an evolutionary rather than revolutionary way. Other competitive factors — such as price and after-sales service — tended to work in favour of larger companies. But because demand for office appliances often outstripped supply, this still left room for smaller companies. A large market share, however, could bring in additional benefits. For example, the sale of punched cards for its machines accounted for nearly 40 per cent of

IBM's revenue in the early 1930s. IBM was also distinguished by its desire to expand the market. By the Second World War, almost all US army and navy accounting used IBM equipment. From well before this, IBM had encouraged the use of its products by scientists and engineers, partly to benefit from technical feedback from these groups.

Cortada believes that the industry rapidly developed patterns of behaviour that then persisted into the era of the electronic computer. For example, there was a strong emphasis on the identification of customer needs and the continuing feedback from the customer. He notes that there were five kinds of customers' queries about a new or modified office appliance: did it do the job better? Did it do the job more cheaply? Was it equally reliable? What other options were available? And could it perform new tasks? From these questions, it is clear why the industry's customer base could readily be persuaded to use electronic computers.

Cortada is primarily concerned with developments in the United States. A company such as Brunsviga, which was well known in Europe, receives only passing mention. Although it would have been interesting to have had more international comparison, US companies were always dominant. NCR, for example, had agents or subsidiaries in at least 15 countries by the 1890s. Data on all these aspects of US dominance — sales, production, profits and so on — are provided in a series of excellent tables; but it is, perhaps, the general points that remain the most interesting. For example, women quickly became involved in using the new office appliances: to what extent was this seen by management as due to a de-skilling of the tasks involved rather than the acquisition of a new set of skills?

IBM long remained highly paternalistic — as exemplified during the Second World War, when wives and dependants of IBM employees in uniform received special financial assistance. This approach to management (including the anthem quoted previously) sounds very similar to the much-publicized managerial activities of today's Japanese companies. Could it be that the old IBM was better able to motivate its staff than the IBM of today? □

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New in paperback

■ Princeton University Press has issued six more classic titles in its Science Library series:

100 Billion Suns: The Birth, Life, and Death of the Stars by Rudolf Kippenhahn, \$20. Contains a new afterword by the author. For a review, see *Nature* **305**, 252 (1983);

Hands by John Napier, revised and introduced by Russell H. Tuttle, \$10.95, £9.95. See *Nature* **288**, 511 (1980);

Laws of the Game: How the Principles of Nature Govern Chance by Manfred Eigen and Ruthild Winkler, \$19.95, £10.95. See *Nature* **298**, 497 (1982);

The Logic of Life: A History of Heredity by François Jacob, \$12.95. See *Nature* **251**, 81 (1974);

Animal Species and Their Evolution by A. J. Cain, \$14.95, £9.95. First published in 1954, and now with a new afterword by the author; and

Chance and Chaos by David Ruelle, \$10.95. See *Nature* **358**, 293 (1992).

■ Penguin has also just published a paperback edition of **Chance and Chaos** at £6.99. Other new popular science titles from Penguin include:

Fearful Symmetry: Is God a Geometer? by Ian Stewart and Martin Golubitsky, £6.99. For a review, see *Nature* **360**, 545 (1992);

The Discovery of Subatomic Particles by Steven Weinberg, £9.99. See *Nature* **308**, 791 (1984);

Dreams of Arizona by Roger D. Stone, a look at the history, ecology, politics and economics of this region, \$13;

The Tangled Wing: Biological Constraints on the Human Spirit by Melvin Konner, £7.99. See *Nature* **301**, 267 (1983); and

The Penguin Dictionary of Science by E. B. Uvarov and A. Isaacs, 7th edn, £6.99.

■ Also of general interest are:

Extinction: Bad Genes or Bad Luck? by David Raup (Oxford University Press, £7.99) and **The Miner's Canary:**

Unravelling the Mysteries of Extinction by Niles Eldridge (Virgin, £6.99). For a review, see *Nature* **354**, 327 (1991);

Narratives of Human Evolution by Misia Landau (Yale University Press, \$14.95, £9.95). See *Nature* **354**, 326 (1991);

Beyond Natural Selection by Robert Wesson (MIT Press, \$14.95, £13.50). See *Nature* **352**, 206 (1991);

Frames of Mind: The Theory of Multiple Intelligences by Howard Gardner (Basic Books, £16). Contains a new introduction by the author. For a review of this hugely influential book, see *Nature* **308**, 792 (1984); and

Women of Science: Righting the Record edited by G. Kass-Simon and Patricia Farnes (Indiana University Press, \$14.95) and **The Outer Circle: Women in the Scientific Community** edited by H. Zuckerman, Jonathan R. Cole and J. H. Bruer (Yale University Press, \$20, £12.95). See *Nature* **354**, 336 (1991).

Gene justice

John F. Y. Brookfield

DNA on Trial: Genetic Identification and Criminal Justice. Edited by Paul R. Billings. *Cold Spring Harbor Laboratory Press: 1993. Pp. 154. \$55.*

DNA PROFILING allows one to establish the source of body fluids left at the scene of a crime with unprecedented accuracy. But there has been considerable debate about the use of such evidence in trial courts, mainly because of the fear that subdivision within racial groups may cause the probabilities of chance matches in DNA profiles to be underestimated. Recent data and analyses have shown that these fears are largely groundless, and DNA identification is now increasingly accepted in US courts.

In 1991, however, a symposium on DNA evidence was instigated by the Human Genetics Committee of the Council for Responsible Genetics, from which this volume now results. Three chapters, by J. Baird, B. Budowle *et al.* and D. A. Berry, strongly support the use of DNA as forensic evidence. These are sandwiched between a collection of chapters in which major misgivings are expressed about its use, particularly in the US criminal justice system.

The principal limitation to criminal justice is that, for most crimes, the totality of evidence available to the investigating authorities is insufficient to establish who commits the crimes. So guilty people are acquitted, or not brought to trial. More rarely, innocent people are convicted because there is insufficient evidence to exculpate them. I take it as axiomatic that, all else being equal, justice is promoted by any technical development that allows courts to find out with increased precision who commits crimes. The use of DNA profiling is one such development, so a criminal justice system is morally required to use DNA information unless there are overwhelming reasons why it should not.

Most of the chapters here suggest such reasons, but fail to convince. Two points made repeatedly must be accepted. First, any technique performed by humans will occasionally be performed carelessly and inaccurately. Second, any technique used by the state to identify criminals could also be used by the state to identify people who are not criminals. While valid, these points are trivially obvious and not specific to DNA. They are dealt with here, in an exaggerated way, by J. S. Kotval and by N. L. Wilker *et al.* Other, even less satisfactory, chapters attempt to raise further concerns. P. R. Billings quotes nineteenth-century

racists and, using a completely opaque argument, seeks to show that the finding of ethnic substructure in DNA profiles inevitably leads to racism. T. Duster contributes a chapter on race that is wholly irrelevant to DNA, while P. L. Bereano's study of government threats to civil liberties is of insufficient quality to warrant detailed discussion.

The crucial chapter is that of M. M. Shultz. Her interesting account concentrates on the interface between science-based systems of identification such as DNA profiling and the US legal tradition. Her view is that the establishment of "substantive truth" (that is, the truth) is not always as important as adherence to traditional procedures by which decisions of guilt or innocence are made in the adversary process. The adversary process with a jury as final arbiter seeks to provide justice, in the form of fairness, in situations where the truth may be obscure. The jury therefore exists not to be expert but to be unbiased, and delivers justice by randomizing the probability of error across defendants in situations where true certainty is impossible. She argues that this function of due process in providing justice amounts to the provision of a different kind of truth, which she calls "procedural truth". The justifications for the primacy of 'procedural truth' lie in the "American commitment to individualism" and in "the frontier spirit".

This is parochial. It is also nonsense. 'Procedural truth' is not necessarily truth and the vision of due process that Shultz advocates is not one that would be generally recognized as providing justice. This is illustrated in her description of a case in which the defence chose not to contest the prosecution's DNA evidence because independent testing for the defence by a different laboratory had shown the evidence to be accurate. She argues that "the role of the defense counsel is not satisfaction by a personal belief that the prosecution's evidence is reliable. The defense counsel has a duty to present a zealous defense. One could argue that this duty requires use of experts to attack the State's DNA testing procedures even if counsel is independently convinced that the results of the State's tests are accurate." The most disturbing aspect of this suggestion is that it exactly describes what defence lawyers have seemed to do in several recent cases in the United Kingdom and in the United States. □

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More models

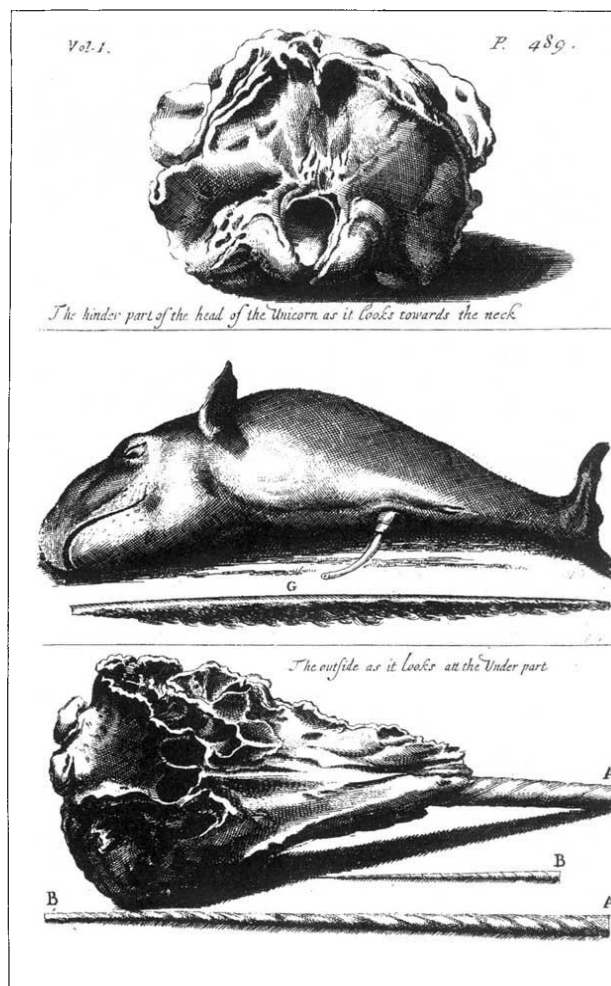
Stuart Sutherland

Creative Cognition: Theory, Research and Applications. By Ronald A. Finke, Thomas B. Ward and Steven M. Smith. MIT Press: 1992. Pp. 239. \$24.95, £22.50.

CREATIVITY, whether in science, literature, music, painting or everyday life remains a mystery, despite the fact that psychologists are increasingly turning their attention to the topic. *Creative Cognition* is not unrepresentative of their efforts. Too often they put old ideas together in imprecise ways, call the result a new theory (or model) and give it a high-sounding name — in the present case “Geneplore”, which competes with such previous expressions as ‘Concept Specialization Model’ and ‘Structural Mapping Theory’. The outcome is usually too commonplace to be new and too vague to be a theory.

R. A. Finke, T. B. Ward and S. M. Smith are no exceptions. They argue that creativity arises from “preattentive structures” that are assembled unconsciously. Most of their examples involve the use of visual imagery. They showed their subjects three or more shapes and asked them to imagine them put together in structures of their own choice. The subjects then had to use their ingenuity to make one of the imagined patterns represent an object of a specified kind (for example, a utensil). For instance, starting with a sphere, a rod and a half-sphere, the subject might picture the sphere connected to one end of the rod with a hollow half-sphere at the other end. The sphere could act as a handle and the half-sphere as a mould to stamp out a hamburger: the subject has invented a utensil for making hamburgers. Independent judges rated the results for their creativity. The more shapes the subject had to choose from and the wider the range of uses to which he could choose to put them, the less creative was the outcome. Does this mean anything more than that the fewer the shapes available to the subject and the smaller the number of uses he is allowed to prescribe, the more he will be forced into allocating a bizarre interpretation of the restricted structures he is permitted to imagine?

The authors call the interpretation of the preattentive structures “the exploratory process”. The process scans the structures “for emergent features”, which in the instance given might allow one to be interpreted as a utensil. At this stage, various operations can be performed on the preattentive structures, such as transforming them, blending



HORNS of a dilemma — for centuries, whalers, explorers and scientists have imagined ever more ingenious uses for the spiral tusk of the narwhal *Monodon monoceros*, the Arctic toothed whale to which the fictional unicorn owes its existence. Herman Melville, amused by all the theories — from ice augur and fish spear to defensive weapon and streamlining device — suggested in *Moby Dick* that the appendage might serve as a letter opener. Modern observations have confirmed that the tusk is used for jousting between rival males. This 1732 drawing showing the skull and horn of the narwhal appears in *The Narwhal: Unicorn of the Sea* by Fred Bruemmer. Swan Hill, £19.95.

them or forming analogies based on them. It is not entirely clear whether the exploratory process takes place at a conscious or unconscious level.

Part of the problem with Geneplore is that in many situations there is no limit to the preattentive structures that might be formed. It is hard to believe that an instant repartee or witticism depends on the examination of many preattentive structures, yet no mechanisms are provided to explain how they are constrained. Moreover, part of human creativity, for example in mathematics, is finding problems that are both soluble and important. There are several computer programs for making deductions, one of which proved most of the theorems in the first part of *Principia Mathematica*, but only at the expense of proving many more that were too trivial to be of any interest. Despite many suggestions, including Herbert Simon's means-ends analysis, there are no satisfactory programs for performing induction: some can, but only in limited domains and only if they are given detailed instructions on how to proceed.

Nothing in this book is sufficiently precise to suggest a working program. The best parts of it are those concerned with well-worn findings. For example,

when someone is trying to solve a problem, the solution often comes out of the blue after a period in which he has not been thinking about it. This phenomenon used to be called ‘the incubation effect’ because it was thought that the unconscious mind was working away at the problem. There is, however, now evidence that if a person is trying very hard to solve a problem, he cannot get away from the idea that is uppermost in his mind; when he ceases to think about it, less obvious ideas are allowed to emerge. Moreover, situations unconnected with work may by association provide the vital clue. If Archimedes had been desk-bound, he might never have had occasion to cry “Eureka”.

The authors believe that creativity can be taught, but do nothing to substantiate this thesis. They recommend potential literary prizewinners to start with a word salad and then try to make sense of it, a prescription that most modern novelists seem to follow without having had the benefit of a creative writing course. One cannot help feeling that there is more to creativity than meets the authors' eyes. □

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Life, the Universe and anything goes

Robert Shapiro

Our Place in the Cosmos. By Fred Hoyle and Chandra Wickramasinghe. Dent: 1993. Pp. 190. £16.99.

IMAGINE that the cosmos teems with life, of a kind similar in biochemistry to our own. Not only are Earth-like planets inhabited, but comets and gas giants as well. Further, the interstellar clouds are composed of frozen bacteria. Life on Earth began when a life-bearing comet arrived from outer space. Further deliveries over several thousand million years have brought viruses, bacteria and even bees here, as a driving force for evolution (Darwin was wrong). All the genes needed for the development of animal life, including our own intellects, arrived in a massive immigration from space 570 million years ago, and waited for a suitable opportunity to express themselves. More recent deliveries have had less kind effects, such as the extinction of the dinosaurs and the initiation of epidemics of bubonic plague, influenza and other diseases.

If this scenario does not strike you as plausible, then you are not alone. For more than a decade, Sir Fred Hoyle and Chandra Wickramasinghe have argued the above to be true in a succession of books (*Our Place in the Cosmos* is the latest) and converted, to my knowledge, no one at all. I choose the word 'converted' with care, for the authors present their case with a degree of inner certainty and in a polemic style that would make a cult leader gnash his (or her) teeth with envy. Their own views are based on evidence that they believe "is irrefutable", while opposing theories are based on "dogma", "without any tangible proof or evidence", and perpetuated by a "cabal" that controls the scientific journals.

This style permeates the presentation, which almost lacks references and is based to a good extent on flawed comparisons and unjustified associations and extrapolations. For example, an almost featureless area of the infrared spectrum from a cosmic source (it contains only broad humps suggesting the presence of C-N, N-H and O-H bonds) is used for an exact identification: "The correspondences between our model and the data are so precise that we have been encouraged to suggest that cosmic dust grains are indeed bacteria". In earlier studies the authors had suggested, with some confidence, that the grains were a silicate-graphite mixture, polyoxymethylene, a mixture of organic polymers, sporopollenin, cellulose, a mixture of polysac-

charides, and then a polysaccharide-hydrocarbon mixture. They do not like to leave a problem unresolved.

Other arguments are weaker still. The ability of bacteria to repair damage inflicted by short-wavelength radiation is cited as proof of their extraterrestrial origins (one might argue as well that their ability to remove bound aromatic amines demonstrates that life began in a dye factory). The discovery of molecules containing two sulphur atoms in comet Halley leads the authors to remark that disulphide links also occur in proteins. The visual discomfort that we experience in bright sunlight suggests to them that the eye evolved in a dimmer setting "of the intensity that would be seen by a person standing on a snowfield on one of the satellites of the planet Uranus".

This book cannot be taken seriously as a

work of science, but it may have other value. Hoyle made noteworthy contributions to physics and astronomy in the earlier parts of his career (though even then, his current ideas were incubating: many appear in his 1957 science-fiction novel *The Black Cloud*). His recent books afford full documentation of the way in which a brilliant mind can be turned to the pursuit of bizarre ideas. One clue to the mechanism is provided by the authors in this very work: "Wherever ultimate origins are concerned, one finds, not the caution and humility that common sense might suggest to be advisable, but an immense show of intellectual arrogance." □

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Climate governed by a child

S. G. Philander

El Niño: Historical and Paleoclimatic Aspects of the Southern Oscillation. Edited by Henry F. Diaz and Vera Markgraf. Cambridge University Press: 1993. Pp. 476. £40, \$59.95.

UNTIL the 1940s, the climate event called El Niño, Spanish for 'The Child' (that is, Jesus), was regarded with joy in Peru and Ecuador when it occurred every few years at Christmas time. Its rains made the local deserts bloom, and the warming of the coastal waters produced treats in the form of unusual marine life. Today we perceive El Niño very differently and associate it mainly with natural disasters on a global scale: devastating floods along the coasts of Ecuador and Peru where the fishing industry is ruined by the disappearance of fish that are usually abundant; droughts in parts of Australia, South-East Asia, India and southern Africa; and unusual weather patterns worldwide, especially over the Americas. Have we changed our perception because of our greater vulnerability to climatic fluctuations? Or has the character of El Niño changed?

This book, a collection of essays by authors who have studied past El Niño episodes, shows that there has probably been little change in the nature of El Niño over the past few millennia, although there is some indication that the frequency of El Niño events increased about 5,000 years ago. Nevertheless, N. Nicholls, in describing how various Australian animals evolved to cope with prolonged droughts associated with El Niño, hints that the events have been with us for longer still. The recent change has been in our perception

of El Niño, which is now a bit too gloomy: in an essay on fishery catch records, G. Sharp reminds us that Peru's lost fish are in fact Chile's gain when they reappear further south in waters that remain cold during El Niño.

Information about past El Niño episodes is available from a variety of other records: Nile flood data (that extend back to 622 AD), tree-ring chronologies, a 206-year record of wild land fires in the southwestern United States, pollen data, ice-core records from tropical and subtropical glaciers in the Andes and Himalayas, coral skeletons that preserve information about oceanic temperatures and salinity, and marine and lacustrine sediments. The extent to which the different records provide information about El Niño varies considerably — there are many factors other than El Niño that affect the climate of the southwestern United States.

The book discusses these matters in detail. To specialists, the articles will be of great value. But nonspecialists may find them too technical and detailed and lacking in background material (although D. Enfield's review is readable and informative). □

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■ CUP has also recently published a paperback edition of *The Earth as Transformed by Human Action: Global and Regional Changes in the Biosphere over the Past 300 Years* edited by B. L. Turner II et al. £27.95, \$39.95. For a review, see *Nature* **355**, 781 (1992).

Anatomy of a basaltic volcano

Robert I. Tilling & John J. Dvorak

Kilauea volcano, in Hawaii, may be the best understood basaltic volcano in the world. Magma rises from a depth of 80 km or more and resides temporarily in near-surface reservoirs: eruption begins when the crust above one of these reservoirs splits open in response to a pressure increase. Repeated rift-zone eruptions compress Kilauea's flanks; after decades of accumulation, the stress is relieved in catastrophic earthquakes and southward displacement of the volcano's south flank.

A PRIMARY goal in studying active volcanoes is to correlate the sub-surface movement of magma with surface and near-surface volcanic activity, not only to understand the volcano's inner workings, but also to detect precursors of impending eruptions. The better we understand how volcanoes work, the better we can mitigate volcanic hazards; yet we are far from achieving this goal, because only a few of the world's 540 or so historically active volcanoes are studied intensively¹. At Kilauea, where eruptions are frequent and accessible², the magma pathway can be defined from source to eruption. Kilauea (Fig. 1), like other oceanic intraplate shield volcanoes, erupts fluid basaltic magma, and hence typically has nonviolent eruptions, which can be studied with minimal danger to observers and scientific instruments. In contrast, nonbasaltic composite volcanoes along convergent plate boundaries, such as Mount St Helens (Washington, United States) and Mount Pinatubo (Luzon, Philippines), erupt viscous magma and typically produce violent, explosive eruptions less amenable to scientific scrutiny.

The scientific literature on Hawaiian volcanism exceeds six thousand references; many recent studies were collected in two special volumes^{2,3}. Here we review the physical aspects of how Kilauea works, drawing mostly from seismic and geodetic measurements; other data are discussed as they relate to magma movement. We will not discuss petrological and geochemical studies of the origin and evolution of Hawaiian magmas, important though these have been to our understanding of the behaviour of the summit magma reservoir and rift zones; characterization of dyke propagation during sub-surface magma movement; estimation of the average magma supply rate of about $0.1 \text{ km}^3 \text{ yr}^{-1}$ and possible variations in this supply rate; and demonstration of a linkage between repeated magma intrusion into the rift zones, mobility of the south flank and the occurrence of catastrophic earthquakes.

The basic elements of a dynamic model of Kilauea (Fig. 2a) proposed in 1960 (ref. 10) still apply. Data acquired from intensive study of later eruptions—including the decade-long and continuing east rift zone activity that began in 1983 (ref. 11)—have added new insights to the 1960 model, including: a sharper definition and refined understanding of the behaviour of the summit magma reservoir and rift zones; characterization of dyke propagation during sub-surface magma movement; estimation of the average magma supply rate of about $0.1 \text{ km}^3 \text{ yr}^{-1}$ and possible variations in this supply rate; and demonstration of a linkage between repeated magma intrusion into the rift zones, mobility of the south flank and the occurrence of catastrophic earthquakes.

The magmatic system

As Eaton and Murata first envisaged, the magmatic system of Kilauea (Fig. 2a) consists of a magma source in the mantle, a vertical pathway by which magma rises to the volcano, a shallow magma reservoir beneath the summit area, and horizontal pathways that transport magma along rift zones radial to the summit area. All historical eruptions have occurred either within the summit area or along a rift zone.

Magma source. In the Eaton–Murata model, which pre-dates

the theory of plate tectonics, Hawaiian volcanism was attributed to 'fundamental instability of the crust and upper mantle' and a regional fracture beneath Kilauea that allowed transport of magma to the surface. Today, it is attributed to magmatic processes resulting from interaction between a 'hotspot' in the mantle and the overriding Pacific plate (Fig. 1a) (see ref. 4). A

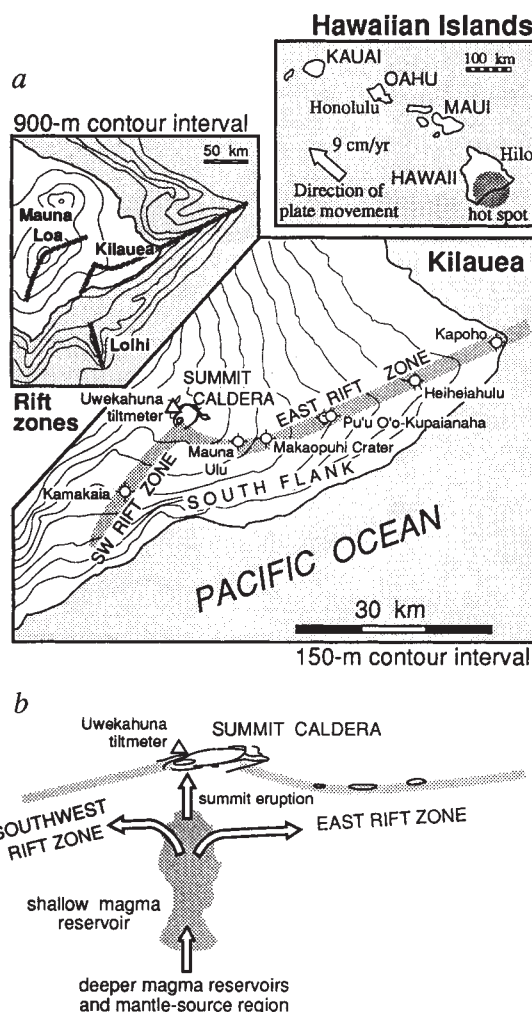


FIG. 1 a, Map of the island of Hawaii showing the three currently active volcanoes—Mauna Loa, Kilauea and Loihi (offshore)—and other features of Kilauea mentioned in the text; prominent rift zones are shown as bands of darker stippling. The generalized location of the Hawaiian hotspot, and the direction and average rate of movement of the Pacific Plate, are indicated in the map of the Hawaiian Islands. b, Schematic cut-away view of the interior of the summit area of Kilauea, showing magma pathways through the summit reservoir. Shown in both a and b is the location of the Uwekahuna water-tube tiltmeter, which has been used to make daily readings of summit tilt since July 1956.

hotspot may be an upwelling thermal plume, which could account for the broad swells and patterns of geoid highs around some intraplate basaltic volcanoes, including those in Hawaii and Cape Verde^{12,13}. According to the hotspot hypothesis, the Hawaiian Ridge–Emperor seamounts originated from the passage of the Pacific plate, at an average rate of about 9 cm yr⁻¹, over a stationary melting anomaly in the upper mantle. Plate movement over the hotspot for more than 70 Myr produced a 6,000-km-long trail of volcanoes—progressively older to the northwest—as each volcano was successively cut off from the feeding magma source, and a new volcano developed.

Magma pathway. Geochemical studies of Hawaiian lavas, and of mantle rocks (called xenoliths) entrained in rising magma, suggest that partial melting of the mantle occurs at depths of ≥ 80 km^{14,15}. The melt zone is well below the 40 km thickness of the elastically defined lithosphere (Fig. 2b), as determined from the pattern of sagging of the Pacific plate by the weight of the volcanoes^{16,17}, and also below the maximum depth of most earthquakes beneath Hawaii¹⁸ (Fig. 3). As magma rises into the brittle lithosphere, it probably collects in pockets, perhaps as melt-filled cracks connected by faults¹⁹ (Fig. 2b).

The magma pathway through the lithosphere to Kilauea is along the pipe-like feature defined by earthquake foci beneath the summit area (Fig. 3). Magma from the region of partial melt apparently is partitioned to individual volcanoes at depths greater than 40 km. Between 20 and 40 km, the feature is about 10 km wide and seems to be inclined slightly to the south; within this depth interval originates a long-period seismic signal called deep volcanic tremor, presumably associated with magma ascent²⁰. Between 7 and 20 km, the seismically defined feature

narrows into a small stem of earthquakes directly beneath the summit and extends part way into the volcano^{18,21}. Above 7 km and extending to within about 2 km of the surface is an aseismic zone²², which is inferred from the lack of earthquakes to be a magma reservoir—a hot, partially magma-filled region too ductile for earthquakes to be produced. Moreover, this zone does not transmit seismic shear waves effectively²² and has a low seismic velocity²³; both attributes are consistent with the presence of a magma reservoir.

Summit magma reservoir. The existence of a shallow magma reservoir within Kilauea, including its possible connections with the rift zones, was suspected by early Hawaiians and surmised in scientific studies as early as 1840 (ref. 24). The general notion stemmed from a near-coincidence of rift-zone eruptions and lowering of a summit lava lake, implying magma withdrawal from the summit area to feed the eruption. A depth of 3–4 km to the summit reservoir was estimated from the pattern of summit subsidence in 1924 (ref. 25) and in 1960 (ref. 26). Similar depths to a magma reservoir have also been determined for some other volcanoes: Mauna Loa volcano²⁷; Piton de la Fournaise, Réunion Island in the Indian Ocean²⁸; Krafla volcano, Iceland²⁹; and Camp Flegrei, the restless caldera near Naples, Italy³⁰.

Analyses of geodetic surveys made in the summit area since 1960 show that loci of maximum uplift or subsidence ('pressure centres') are within the 2–7 km depth interval of the summit aseismic zone (Fig. 4). Apparent shifts in the location of pressure centres during and between eruptions, most clearly reflected in systematic changes in direction of daily summit-tilt measurements³¹, suggest that the summit reservoir is not a single cham-

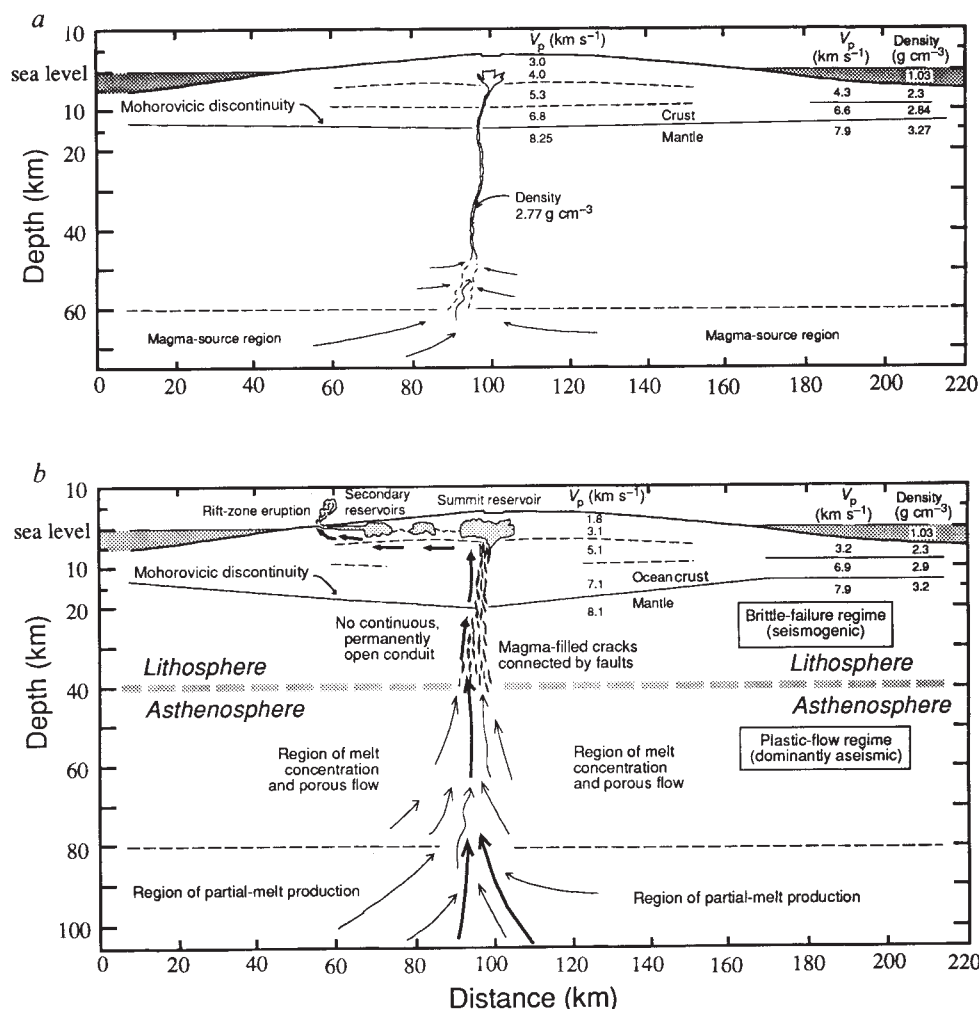


FIG. 2 a, Dynamic model of an idealized Hawaiian volcano, slightly modified (to depict without vertical exaggeration) from Figure 5 of Eaton and Murata¹⁰, who suggested that "magma from a source about 60 km deep streams up through permanently open conduits and collects beneath the caldera" (see text for details). b, Updated model that reflects refinements to the Eaton–Murata model; seismic velocities and densities are taken from ref. 59. See text for discussion. v_p is the velocity of seismic compressional (primary) wave.

ber, but a plexus of interconnected magma pockets³², perhaps a network of dykes and sills. This idea is also supported by a unique and uniform composition ('magma batch') for each historical summit eruption, suggesting that only one magma pocket—or perhaps a group of well mixed, connected pockets—is tapped and feeds that particular eruption³³. Recently, Yang *et al.*³⁴ showed that the vertical and horizontal ground displacements for both inflation and deflation episodes between 1970 and 1985 could be modelled by a combination of a single, stationary magma body and (for the inflation episodes) radial dykes. They questioned the migration of pressure centres, suggesting that the apparent shifts in position reflected the inadequacy of point-source models. However, their single-magma-body model is also deficient in not considering the systematic change in tilt direction at Uwekahuna (Fig. 1) as magma fills, and then withdraws from, the summit reservoir³¹. The answers to the questions of whether Kilauea's summit reservoir is single or multiple, and stationary or mobile, must await additional data and more comprehensive models. What is indisputable, however, is that magma is stored predominantly beneath the southern part of the summit region³², in the area defined by the cluster of pressure centres in Fig. 4 and the single magma body of Yang *et al.*³⁴.

Changes in the volume of magma in the summit reservoir can be estimated from the rise or fall of the volcano's surface as magma enters or leaves. This approach assumes the volume of surface deformation to be equal to the change in volume of magma stored within the reservoir, an assumption supported by comparison of volumes of lava erupted with volumes of summit subsidence³⁵, and material injected into the crust with resultant uplift³⁶. Since 1959, the change in volume of magma in the summit reservoir has been at most 0.4 km³, and magma storage in the reservoir was greatest during the mid-1970s³⁷. Before 1959, volcano-wide geodetic surveys were infrequent (only two surveys in the 1920s and one in 1958) although summit-tilt readings have been made daily since 1913. From the sporadic pre-1959 measurements, the elevation of a benchmark in the south summit area, near the point of maximum elevation change as the summit area rises or falls, is now more than 4 m lower than it was in 1920, which corresponds to a net volume decrease of 0.8 km³ of the summit reservoir³⁸. Daily tilt readings suggest that the summit reservoir, which was at its most swollen in 1920 and nearly regained that state by 1974, is now at its least swollen state since 1913.

Rift zones and secondary magma reservoirs. Two shallow, seismically active regions lead from the cluster of summit pressure centres, probably tracing the beginnings of horizontal pathways that transport magma from the summit reservoir to the two rift zones (Fig. 4). The summit reservoir can directly feed some rift-zone eruptions, especially into the southwest rift zone^{39,40}, or feed secondary magma reservoirs (Fig. 2b) beneath a rift zone³³. Localized ground deformation along the east rift zone marks the presence of secondary magma reservoirs (Fig. 1a), one beneath Makaopuhi crater^{41,42} and probably about 3 km down⁴³; another near Heiheiiahulu³⁵; and a third suspected near Kapoho crater, as the probable initial source of lava during the 1955 and 1960 rift-zone eruptions³³. A small secondary reservoir may lie beneath the southwest rift zone near Kamakaia (Fig. 1b), suggested by repeated magma intrusions during 1981–82 (ref. 18). Concentrations of shallow earthquake swarms between secondary reservoirs may indicate barriers to horizontal flow of magma¹⁸. Barriers may be posed by structural features along a rift zone, such as a bend in the east rift zone near Mauna Ulu³⁹ or an offset near Heiheiiahulu, or may be temporary obstructions that are cleared by repeated magma flow⁴⁴.

The scarcity of earthquakes at depths greater than 5 km beneath the rift zones suggests that these zones do not extend into the ocean crust, but develop upward as the volcano grows. The active core of a rift zone lies within a few kilometres of the surface. Repeated magma injections as vertical dykes into this

core may feed an eruption and create normal faults and fractures⁴⁵. Delaney *et al.*⁴⁶ suggested that pressurization of a large magma body beneath the summit area and along the rift zones at a depth of 3–9 km produced the pattern of vertical and horizontal movements across the east rift zone and along the south flank from 1976 to 1989. Dvorak *et al.*⁴⁷ offer an alternative interpretation: rates of seismicity and ground movements during 1976–89 may reflect the growth of recent eruptive fissures and relaxation of seaward displacement of the mobile south flank after the magnitude-7.2 Kalapana earthquake in November 1975 (ref. 48).

How the volcano works

The classic work of Eaton and Murata¹⁰ not only elucidated the basic configuration of Kilauea's magmatic 'plumbing' system, but also provided the impetus to refine volcano monitoring techniques so as to be able to track the movement of magma through the system. Subsequent studies have shown that all magma ascending from the mantle source region is stored for a finite time in the summit reservoir, and that the above-described components of the magmatic system work in concert. Activity involving one component may affect activity in other

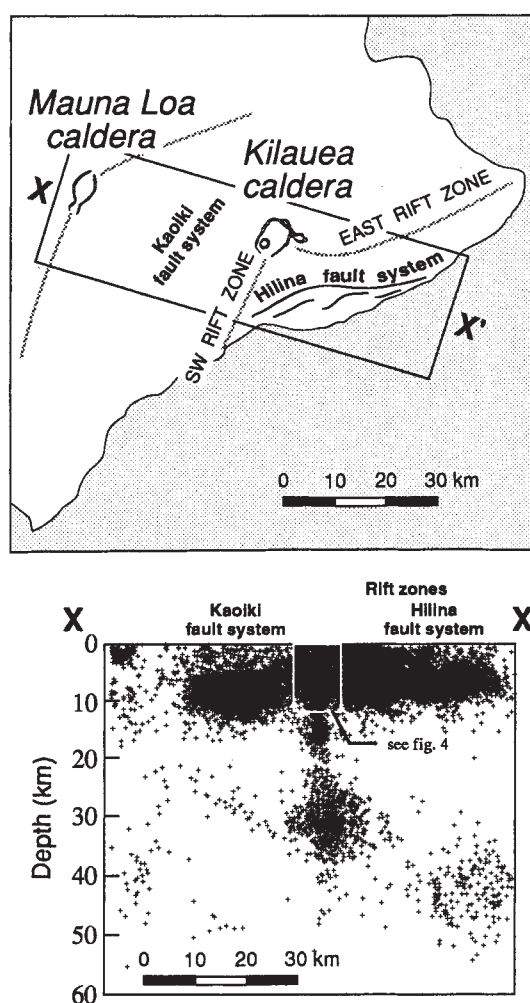


FIG. 3 Earthquake pattern beneath Kilauea from 1970 to 1983 (modified from ref. 18); we selected only earthquakes located within the rectangle outlined in the upper diagram. In addition to the summit calderas and rift zones of Mauna Loa and Kilauea, the locations of two major fault systems are also shown. In the lower diagram, earthquake locations are projected onto a vertical plane X–X' running west-northwest to east-northeast; the area outlined by a white-line rectangle is shown in greater detail in Fig. 4. See text for discussion.

components: for example, the rupture of a secondary magma reservoir may lead to a rift-zone eruption, which simultaneously withdraws magma from the summit reservoir and compresses the adjacent flanks. In turn, magma withdrawal may cause a temporary increase in magma supply from the mantle. Repeated magma intrusions into a rift zone can drive the unbuttressed south flank of Kilauea seaward, setting the stage for catastrophic earthquakes and massive landslides, which may affect the evolution of both the subaerial and submarine parts of the volcanic edifice.

Magma ascent from the mantle. Once a partial melt has formed, presumably in response to pressure reduction as solid mantle material ascends in the hotspot plume⁴⁹, a liquid fraction separates by porous flow through the unmelted residue⁵⁰ and rises by buoyancy or by interstitial pressure exerted on solid grains.

Eaton and Murata¹⁰ suggested that magma rose from the mantle through "permanently open conduits", although the existence of such conduits has been questioned⁵¹. Instead, once rising melt has accumulated into a body of sufficient size, perhaps a few hundred metres in vertical extent, buoyancy forces may exceed fracture resistance of the surrounding rock⁵², allowing the body to ascend as a vertical, lens-shaped crack by opening fractures ahead of it^{19,35}. These bodies of melt may correspond to the chemically distinct magma batches identified in summit lavas³³.

Stress concentrations at the crack tip may produce shear failure, so that the system operates as a network of magma-filled cracks connected by faults^{19,54}. Magma ascends in short bursts, and the movement of one magma-filled crack causes nearby cracks to adjust, analogous to the spasmodic advance of cars enmeshed in a heavy traffic jam. Because magma rises by buoyancy, it may become trapped against the base of the ocean crust, a density boundary⁵⁵. Geochemical studies of Hawaiian xenoliths⁵⁶ are consistent with an interpretation that magma accumulates at the base of the ocean crust—a process called underplating⁵⁷. Underplating may have resulted in a 4-km-thick body beneath the island of Oahu⁵⁸, although a similar-sized body has not yet been formed beneath the island of Hawaii⁵⁹.

Magma continues to rise through the ocean crust and into the volcano until it reaches a horizon of neutral buoyancy⁶⁰, where the magma density equals that of the surrounding solid rock. For a basaltic volcano, this horizon lies at about 3 km below the surface, which matches well the depth of the top of the summit reservoir at Kilauea, as well as other basaltic volcanoes such as Mauna Loa²⁷, Krafla, Iceland⁶¹ and Piton de la Fournaise²⁸.

Behaviour of the summit reservoir. All magma erupted at Kilauea probably first passes through the summit reservoir, as implied by the temporal coincidence between a rift-zone eruption or intrusion and summit subsidence (Fig. 5a). Other evidence includes compositional differences between summit and rift-zone lavas, interpreted as mixing of magma that supplied different summit eruptions and cooler, more differentiated magma stored beneath a rift zone³³. That all magma from depth spends some time in the summit reservoir is suggested also by volcanic-gas studies, in particular, the distribution of carbon and sulphur emissions^{62,63}. A carbon/sulphur molar ratio of 2 to 4 typifies gases from fresh magma, not previously degassed, that recently rose from the mantle, whereas ratios of 0.2 to 0.3 reflect prior escape of an immiscible CO₂-rich fluid phase from gas-saturated magma that has been stored at shallow depth. Some gas samples collected in the summit area have C/S ratios of 2, indicating minimal degassing and short residence time in the shallow reservoir, but most have ratios closer to 0.2 and therefore longer residence time. All samples collected along the rift zones, however, have ratios of about 0.2, as would be expected from magma that first was stored and degassed in, then passed through, the summit reservoir.

Caldera development and explosive eruptions. As has long been recognized²⁴, a close relation probably exists between movement of magma through the summit reservoir and development of the summit caldera. Withdrawal of a large volume of magma from the shallow reservoir caused the unsupported roof to collapse along ring faults, forming the caldera. The formation was not necessarily the result of only a few catastrophic summit collapses, but may have occurred incrementally by smaller collapses over different portions of the reservoir⁶⁴. Incremental growth has also been suggested for other volcanic calderas⁶⁵. A key question is: are large summit collapses associated with rare explosive eruptions in the summit area?

The area beyond the caldera rim and small patches of the caldera floor are not covered by lava flows, but by volcanic ash, as much as 11 m deep⁶⁶. The ash is draped unbroken across arcuate faults that form the caldera, indicating that fault movement came before deposition of the ash, produced by an explos-

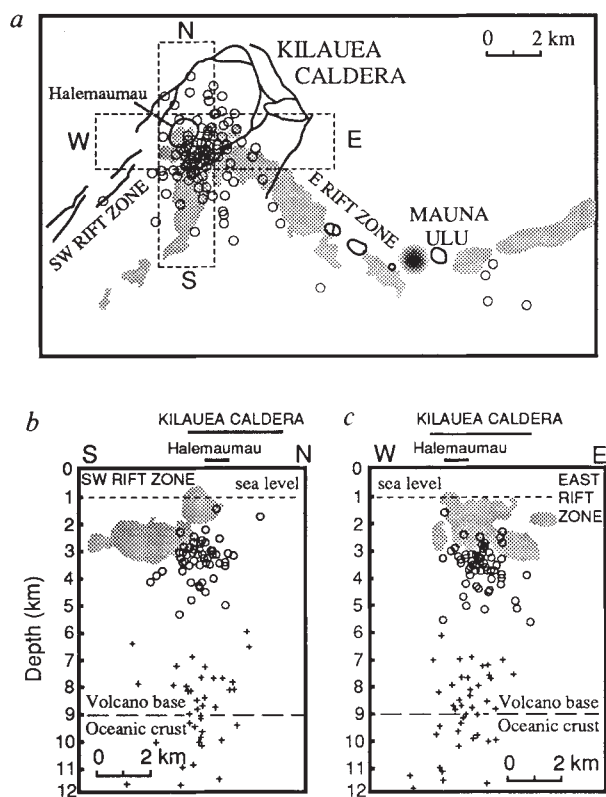


FIG. 4 Location and configuration of the summit magma reservoir at Kilauea. *a*, Plan view. Major vertical faults that define the summit caldera and the upper segment of the southwest-rift zone are shown as solid lines. Halemaumau is a pit crater within the caldera which has been the site of long-lived, active lava lakes since at least the 1820s. Mauna Ulu is a basaltic shield that developed during prolonged activity from May 1969 to July 1973 (ref. 68, 69). Intense epicentral zones of short-period, shallow earthquakes are indicated by a stippled pattern (earthquake patterns adapted from ref. 18). Open circles are locations of pressure centres determined from tilt or levelling surveys conducted between 1958 and 1992. Most pressure centres lie near the intersection of the two shallow earthquake zones leading into the rift zones. Most magma storage occurs in the region marked by the densest clustering of pressure centres, beneath the southern part of the caldera. *b*, North-south cross-section through the summit area of Kilauea. Plus symbols are locations of long-period, deep earthquakes recorded between 1970 and 1983. Depths are relative to the summit region, which has an average elevation of about 1 km above sea level. The magma reservoir is inferred to lie within the aseismic region between shallow and deep earthquake zones. *c*, West-east cross-section through the summit area of Kilauea. Pressure centres lie within the upper half of the aseismic region and are nestled next to the zones of shallow earthquakes. The base of Kilauea is estimated to be about 9 km deep beneath the summit area²⁷, consistent with models of island-wide seismic refraction and gravity surveys⁵⁹.

ive eruption in 1790 (ref. 67). The volatile content of Hawaiian magmas is too low to generate sufficient gas pressure for explosive, ash-producing eruptions; instead, rapid inflow of perched groundwater into hot rock—and perhaps direct contact with magma—may have triggered explosive boiling of the groundwater⁶⁷. Inflow of ground water may have occurred during a sudden lowering of the magma column, such as during the withdrawal of magma accompanying an eruption along the undersea extension of the east rift zone^{66,67}. The 1790 ash contains a significant amount of fresh-appearing glass, suggesting that magma was involved in the production of the ash⁶⁶. Explosive activity at Kilauea may also have resulted from the eruption of magma through a shallow aquifer, perhaps when the caldera floor was a few hundred metres deeper than today, and thus closer to the top of the regional water table. In contrast to the 1790 ash, ejecta hurled by the steam explosions in May 1924 from Halemaumau (Fig. 4a) were composed entirely of older volcanic rock and contained no new magma. The 1924 explosions, much smaller than the 1790 explosions, occurred three months after draining of an active lake in Halemaumau⁶⁷, suggesting that magma had already been withdrawn from shallow levels before groundwater encountered hot rock.

Summit-tilt variations. The typical pattern of slow rise and rapid subsidence of the summit area is shown by ground-tilt measurements for nearly all Kilauea rift-zone eruptions (Fig. 5a).

Magma rises for several months to a year into the summit reservoir, causing the surface of the volcano to rise gradually, 10 to 100 times more slowly than the rate of surface subsidence when magma moves laterally from summit reservoir to rift zone (compare timescales and tilt rates in Fig. 5a and b). During long-lived rift-zone activity, however, such as the 1969–74 Mauna Ulu eruptions^{68,69} or the decade-long and continuing Pu'u O'o-Kupaianaha eruption^{11,70}, vertical magma influx into the reservoir nearly matched the rate at which magma flowed into a rift zone. In the first years of these long-lived eruptions, activity was episodic along the rift zone; each episode was preceded by a few weeks of slow summit uplift (Fig. 5c) and coincided within a few hours to several days with summit subsidence (Fig. 5d).

During uplift, the rate of shallow summit earthquakes generally increased, whereas long-period, deeper earthquakes occurred at the end of and after each eruptive episode (Fig. 5c). Shallow earthquakes are related to extension and cracking of the brittle cap over the magma reservoir, whereas long-period, deeper earthquakes are probably related to rapid refilling of the reservoir from below¹⁸. Similar patterns of seismicity have also been observed before and after the 1984 eruption of neighbouring Mauna Loa⁷¹ and the 1980 explosive eruptions of the dacitic volcano Mount St Helens^{72,73}.

Because magma is a fluid, it flows in response to a pressure

FIG. 5 Tilt measurements in the summit area of Kilauea (see ref. 110 for description of techniques used to measure ground displacements at Kilauea).

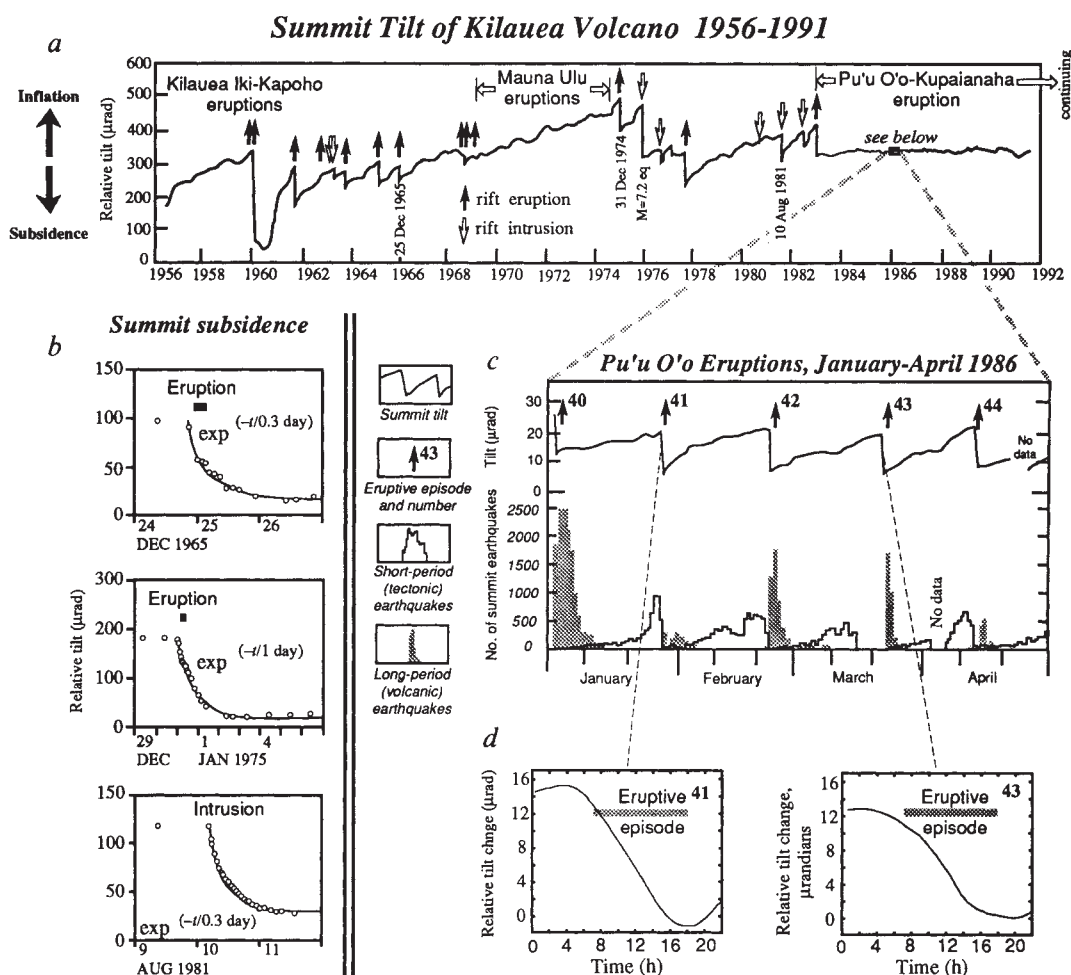


FIG. 5 Tilt measurements in the summit area of Kilauea (see ref. 110 for description of techniques used to measure ground displacements at Kilauea). **a**, East-west component of daily tilt measurements made by the water-tube instrument at Uwekahuna (see Fig. 1). A higher tilt value corresponds to uplift of the surface and, generally, inflation of the summit reservoir. A sudden tilt change of 296 microradians, caused by a magnitude 6.6 earthquake on 16 November 1983, along the Kaoiki fault system has been removed. On average, a change in tilt of 1 μrad at Uwekahuna corresponds to $\sim 5 \text{ mm}$ of uplift in the southern part of the summit caldera. Rift-zone eruptions of major intrusions are indicated by small vertical arrows; a minor summit eruption also occurred during the large subsidence and rift-zone intrusion in November 1975. Major rift-zone intrusions were identified by the occurrence of shallow earthquake swarms somewhere beneath a rift zone and the simultaneous occurrence of at least 10 μrad of tilt change recorded at Uwekahuna¹⁸. **b**, Frequent tilt measurements of the water-tube instrument reveal an exponential decay in the rate of tilt change during summit subsidence accompanying eruptions in December 1965 and December 1974, and an intrusion in August 1981; solid bar indicates duration of eruptive activity at vent. **c**, East-west tilt changes and summit earthquake rates during four months of episodic

eruptive activity along the east rift zone. These measurements were made by a continuously recording tiltmeter in use at Uwekahuna since 1967. **d**, Each eruptive episode from January 1983 to June 1988 coincided with subsidence of the summit area; shaded bar indicates duration of eruptive activity at vent.

gradient. Its ascent from the mantle to the summit reservoir is driven by the pressure gradient imposed by gravity acting on the density difference between lighter magma and heavier surrounding rocks³¹. Magma rises above the summit reservoir when the internal pressure of the reservoir is sufficient to wedge apart the roof rocks and force magma to the surface. Horizontal movement of magma within the rift zones is driven by gravity and the pressure gradient imposed by the slope of the volcano, so that larger volumes of magma are withdrawn when eruptions occur at lower elevations⁷⁴. Pressure-gradient control of magma movement is evident also as the exponential decay in summit tilt (Fig. 5b) as magma moves into or out of the summit reservoir⁷⁵, as well as in the exponential decay of eruptive rate during the 1944 eruption of Vesuvius⁷⁶ and several eruptions of Etna⁷⁷; similar patterns of surface movement have been recorded at Krafla, Iceland⁷⁸.

The regular rate of summit subsidence at Kilauea (Fig. 5) may furnish a basis for predicting the duration and volume of the subsidence in progress, by real-time extrapolation of rates of summit-tilt change shortly after onset of subsidence. More important, if an empirical relationship can be established between summit subsidence and rift-zone activity, then it may be possible to predict the duration and volume of an accompanying eruption. For example, during the 19 eruptive episodes of Pu'u O'o from February 1983 to June 1984, the beginning of almost all episodes lagged the start of summit subsidence by a few hours and continued for a few hours after it had stopped (for two examples, see Fig. 5d)⁷⁰. Moreover, long-period earthquakes at depths of 5–13 km also augured the end of each eruptive episode; such occurrences also have been observed at the end of other summit subsidences and possibly reflect rapid refilling of the summit reservoir¹⁸. Since 1983, such information has been used informally to advise local Civil Defense officials about the progress of an ongoing eruption and its expected end. **Magma supply rate.** Because all magma supplied to Kilauea seems to pass first through the summit reservoir, magma supply rate can be estimated by knowing the volume of erupted lava and the change in volume of magma stored in the reservoir. For example, three long-lived eruptions in 1952, 1967–68 and 1969–71, each characterized by a negligible net change in volume of the summit reservoir, yielded an average magma supply rate of $0.1 \text{ km}^3 \text{ yr}^{-1}$ (ref. 79). Subsequent long-lived eruptions during 1972–74 and 1983–84 indicated nearly the same supply rate^{69,70}. At other times, the supply rate may be different, for example during the initial rapid refilling of the summit reservoir after withdrawal of magma to feed a rift-zone eruption, as revealed by tilt measurements^{31,33}.

Since 1958, the volume of erupted lava, about 2 km^3 (refs 70, 80) has considerably exceeded the maximum change in volume of magma in the summit reservoir of 0.4 km^3 (ref. 37). This means that, as a long-term average over a period of several eruptions, eruptive rate is indeed a measure of supply rate. Between 1958 and 1984, the average eruptive rate was $0.08 \text{ km}^3 \text{ yr}^{-1}$, whereas the average eruptive rate between 1840, the first estimation of the volume of a Hawaiian lava flow, and 1958 was $0.008 \text{ km}^3 \text{ yr}^{-1}$, a factor of 10 less and close to the eruptive rate of about $0.01 \text{ km}^3 \text{ yr}^{-1}$ estimated⁸¹ for the entire Hawaiian-Emperor volcanic chain, averaged over the past 70 million years. The recognition of 'prodigious' submarine landslides⁹ requires this long-term average rate to be a minimum estimate. In any case, the eruptive rate and, by implication, the supply rate during the past few decades seem to be considerably higher than in the past. Because most eruptive activity in the nineteenth and the first half of the twentieth century was confined to the summit area, this apparent increase in supply rate seems to be related to frequent eruptive activity along the rift zones³⁷.

Another indicator that supply rate might be related to eruptive activity is an increase in deep volcanic tremor after the start of long-lived eruptions, which formed Mauna Ulu³⁵ and Pu'u

O'o-Kupaianaha⁸². Deep tremor may be caused by magma flow near the base of the lithosphere²⁰; if so, the year or so lag between the start of both long-lived eruptions and the increase in tremor may represent the amount of time needed for the magmatic system at the base of the lithosphere to respond to surface activity.

Start of a typical eruption. An eruption of Kilauea typically begins by rupture of one of its shallow magma reservoirs, in a manner probably analogous to hydraulic fracturing of wall rock in a borehole by increasing fluid pressure⁸³. Pressure may increase by addition of more magma or exsolution of volcanic gases as the magma cools. Whatever the mechanism, cracks form and propagate in the rock, providing potential avenues, for injection of magma. If magma reaches the surface, an eruption ensues. Some investigators also relate eruption onset to dyke instability^{45,53}.

As an example, seismometers and tiltmeters were used to monitor the growth of a magma-filled crack that in January 1983 initiated the decade-long and continuing east rift zone eruption at Kilauea¹¹. A shallow swarm of earthquakes began near the secondary magma reservoir beneath Makaopuhi crater. During the next 16 hours, the zone of shallow earthquakes extended 10 km along the rift-zone axis, directed away from the summit area, at an average growth rate of 600 m h^{-1} (ref. 84). Earthquakes persisted for eight more hours, ending when magma breached the surface and an eruption began. A tiltmeter located a few hundred metres north of the eventual eruptive vent recorded the wedging open of the rift zone, which was initiated about 2.5 km beneath Makaopuhi crater when the earthquakes began⁸⁵. The crack grew horizontally at an average rate of about 600 m h^{-1} , same as the earthquake migration rate, and upward at an average rate of 70 m h^{-1} . At the end of this event, magma was injected along 12 km of the rift zone, widened the rift zone by an average of about 3 m, and caused a temporary increase in earthquakes beneath the south flank, reminiscent of an after-shock sequence (Fig. 6a)⁸⁶.

More magma is partitioned into, and erupted from, the east rift zone than the southwest rift zone^{35,39}. The relative dimensions of dykes that have formed in each rift zone suggest that a greater magma driving pressure is required to form a dyke in the east rift zone^{40,45}, so that, as stated by Duffield *et al.*³⁹, "...dyke emplacement into the southwest rift zone may be relatively passive compared to the east rift zone." Less frequent activity in the southwest rift zone may be because it is closer to neighbouring Mauna Loa, which restricts movement of the north flank of Kilauea³⁹. After several intrusions into or eruptions from one rift zone, which may last several months to a few years, activity switches to the other rift zone, then back again¹⁸. How quickly this switch may take place is illustrated by one example: when activity switched from the east to the southwest rift zone in January 1981, summit earthquake mechanisms suggest that reorientation of the local stress field occurred only a few days before the switch of activity to the southwest rift zone⁸⁷.

Mobile south flank and catastrophic earthquakes. The widening of the east rift zone in January 1983 compressed the adjacent segment of the south flank (Fig. 6a). This event was not unique: thousands of dykes have resulted in a continuing widening of the rift zone. But because Kilauea's north flank is buttressed by the adjacent volcano Mauna Loa, widening has not been symmetrical about the rift-zone axis, but directed southward and seaward. In repeated geodetic surveys, Swanson *et al.*⁴² recognized strain accumulation along the south flank, which led them to predict a subsidence event along the Hilina Pali fault system (Fig. 3)⁴². While their paper was in press, such an event occurred, causing the magnitude 7.2 earthquake in November 1975, which caused subsidence and southward displacement of the coast of as much as 3.5 and 8 m, respectively^{48,88}. A catastrophic earthquake in April 1868, which affected the south flanks of both Kilauea and Mauna Loa, may have had a similar origin⁸⁹. The cumulative effect of many such events may have formed

the kilometre-high, seaward-facing scarps along the south flank.

Ando⁹⁰ and Nakamura⁹¹ suggested that compression of the south flank is relieved by seaward sliding of the base of Kilauea, perhaps along a low-strength layer composed of ocean sediments sandwiched between the ocean crust and the volcano⁹². This layer may have been detected by reflected seismic phases⁹³. In contrast, Swanson *et al.*⁴² suggested that strain release and sub-surface slip occur over range of depths, so that the south flank behaves as a massive elastic spring, compressed by rift-zone intrusions which may feed eruptions, and released by an occasional destructive earthquake. Such a compression-and-release cycle may be indicated by the rift intrusions and associated eruptions that began in 1977 and 1983, followed by a magnitude-6.1 earthquake beneath the south flank on 26 June 1989 (Fig. 6)⁹⁴. An analogous relation of rift-zone activity and flank earthquakes might also produce devastating earthquakes along the west flank of Mauna Loa, such as the magnitude 6.7 Kona earthquake in 1951 (ref. 95).

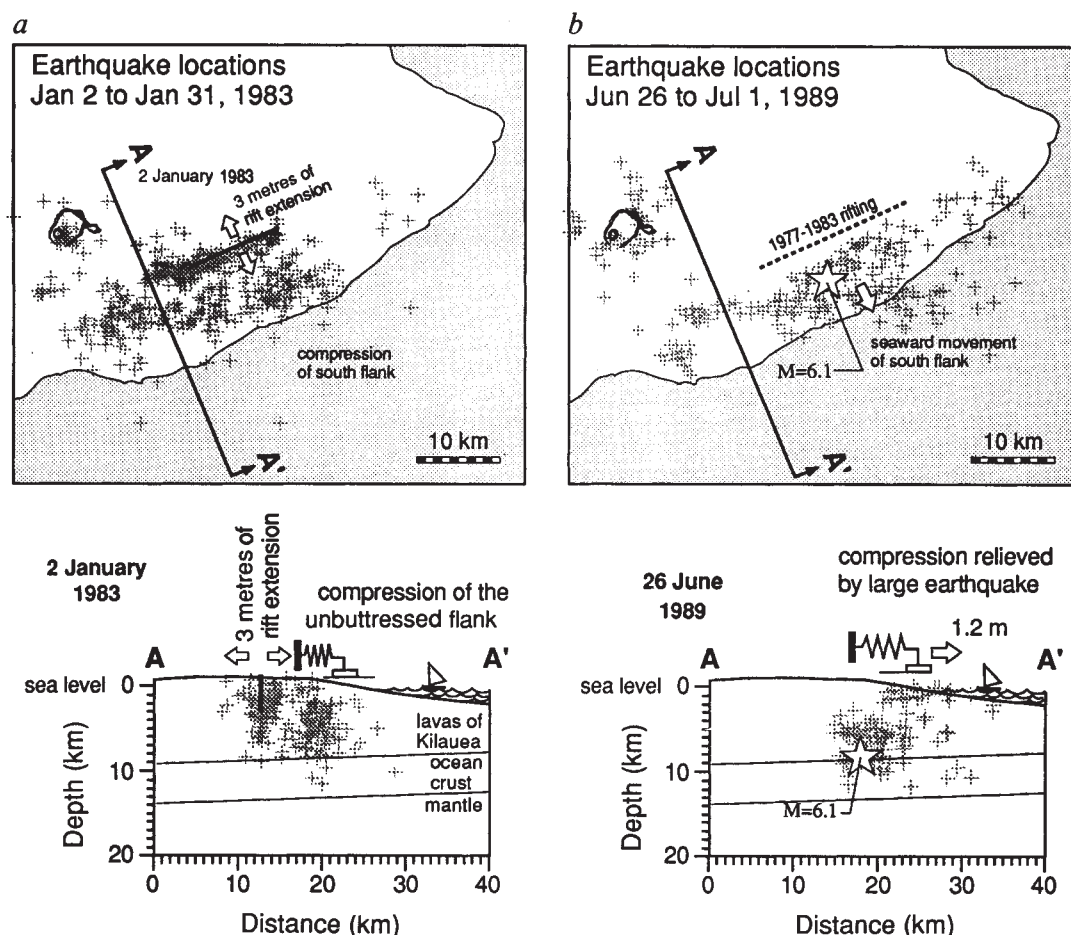
After the magnitude 7.2 Kalapana earthquake in November 1975, seismicity along Kilauea's vertical magma conduit decreased abruptly, indicating that the 1975 earthquake relieved stress on this part of the magmatic system¹⁸. But no obvious effect was observed for shallow seismicity beneath the summit and the adjacent upper east rift zone. Apparently, shallow earthquakes mainly reflect local stress induced by magma pressure, whereas deeper parts of the magmatic system are influenced more by regional stresses. The Kalapana earthquake also reduced the incidence of rift eruptions for nearly a decade⁹⁶, as evidenced by an increased ratio of intrusions to eruptions after 1975 (Fig. 5a). Subtle changes in trace-element patterns of lava also suggests that large flank earthquakes can perturb the magmatic system, as suggested for Mauna Loa following a catastrophic earthquake and associated eruptions⁹⁷.

Post-1960 advances and future research challenges

Our knowledge of the magmatic system of Kilauea has advanced substantially since the ideas put forth by Eaton and Murata¹⁰, mostly through intensive measurement of seismic signals and ground movements associated with its frequent, and often long-lived, activity since 1960. Significant advances include: (1) a detailed delineation of the upper part (≤ 20 km) of Kilauea's plumbing system and the development of a capability to monitor, in near real-time, sub-surface magma movement; (2) a refined understanding of the location and behaviour of the summit reservoir and secondary reservoirs beneath the rift zones, including the observation that pressure centres of ground uplift or subsidence can appear to shift within an area of several square kilometres over the southern part of Kilauea caldera; (3) the detailed characterization of dyke propagation during magma movement; horizontal propagation rates are typically several hundred metres per hour; (4) an estimation of Kilauea's current average magma supply rate ($\sim 0.1 \text{ km}^3 \text{ yr}^{-1}$) from analysis of lava erupted and change in volume of the summit reservoir during long-lived activity; the supply rate may vary on timescales from 10^6 to 10 years; and (5) precise geodetic and seismic documentation of the dynamic interaction between repeated magma intrusions into the rift zone, seaward movement of Kilauea's south flank displacements on the Hilina fault system (Fig. 4), and occurrence of catastrophic earthquakes.

Nevertheless, our understanding of Kilauea's shallow plumbing is still limited, because most measurements are restricted to the summit area or to short segments of the rift zones. The recent advances highlighted above are still controversial and need to be tested by continued comprehensive studies. The most compelling need is to make measurements during the few hours when a dyke grows and magma moves rapidly from a sub-surface reservoir to another reservoir or to feed an eruption. During

FIG. 6 Cross-sections of the east rift zone and south flank, illustrating the dynamics of recurring dyke intrusions which compress the flank, and sudden, seaward movement during large flank earthquakes. *a*, On 2 January 1983, a 12-km-long, 3-km-high dyke wedged open a segment of the east rift zone an average of 3 m and compressed the adjacent part of the south flank⁸⁶. *b*, On 26 June 1989, a magnitude-6.1 earthquake caused the same part of the south flank to move seaward by about 1.2 m, possibly relieving the compression caused by rifting episodes and eruptive activity in September 1977 and January 1983 (ref. 94).



such a short period, it is necessary to make simultaneous measurements at many sites of tremor amplitude and frequency and of slower ground movement to reveal the rate and amount of magma movement. Tremor studies require closely spaced arrays of seismometers that can distinguish surface-wave patterns set up by different tremor sources⁹⁸. Ground-movement studies require a reliable system that can record, at centimetre-scale resolution, three dimensional displacements at least every few minutes, as might be provided by the satellite-based Global Positioning System (GPS)⁹⁹. Also, operation of strainmeters, a few hundred metres below the surface in boreholes will allow sensitive measurements of the three-dimensional pattern of crustal movement. Such measurements have already begun at Long Valley caldera, California¹⁰⁰, and Izu-Oshima Volcano, Japan¹⁰¹.

At present, our knowledge of the deeper (>20 km) magmatic system beneath Kilauea is poor. We lack definitive answers to many crucial questions. For example, what controls the rate of partial melting? How and at what rate does magma ascend from the melt generation region below 80 km? Are these processes continuous or episodic? How are they influenced by interaction between the Pacific plate and the Hawaiian hotspot? Can we quantify the relations among massive slope failures, catastrophic earthquakes, and dynamic behaviour and structural evolution, as proposed for Mauna Loa and other volcanoes along the Hawaiian ridge^{9,102,103}? Because the Hawaiian Islands represent only the small subaerial parts of a huge submarine volcanic chain, future research must range farther afield and deeper. Specifically, we must expand our investigations of the submarine extensions of the volcano's flanks and rift zones. Exploration of the sea floor, using advanced imaging systems and submersible diving vessels, has already revealed that submarine lava fields, massive landslides, and drowned and tilted reefs occur on a scale that dwarfs similar phenomena seen on land or near shore^{9,104,105}. The chemical evolution of basaltic lava over the entire history of a volcano may be better understood by the end of the decade, if plans to drill and core through the ocean crust near Hilo, Hawaii¹⁰⁶, are carried out.

Thomas Jaggar's aim in establishing a permanent volcano observatory at Kilauea in 1912 was not only to conduct research on volcanic phenomena, but also to apply the scientific knowledge gained to reduce volcanic risk and human suffering worldwide¹⁰⁷. His purpose is no less important today, when population growth has made hundreds of millions of people vulnerable to potentially dangerous volcanoes¹. Our improved understanding of Kilauea, and the methods used to gain it, have been transferred to studies of other volcanoes, basaltic and non-basaltic, such as at Mount St Helens in 1980 (ref. 108) and at Mount Pinatubo in 1991 (ref. 109). As convincingly demonstrated at Pinatubo, fatalities and economic loss from a huge eruption—one of the largest this century—in a densely populated area can be greatly reduced with timely scientific response and effective action by emergency-management authorities. □

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***C. elegans* lin-45 raf gene participates in let-60 ras-stimulated vulval differentiation**

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Vulval differentiation in *Caenorhabditis elegans* is controlled by intercellular signalling mediated by a receptor tyrosine kinase and a *ras* gene product. The *lin-45* gene encodes a homologue of the *raf* family of serine/threonine kinases and is necessary for vulval differentiation. The *lin-45 raf* gene product appears to act downstream of the *ras* protein in this pathway. A proto-oncogene-mediated signalling pathway may be a common feature of metazoan development.

DURING *C. elegans* vulval induction, the anchor cell of the gonad induces three of six ectodermal precursor cells to generate vulval tissue¹. The other three cells generate nonspecific epidermis (Fig. 1a). Several genes necessary for vulval induction encode proteins homologous to mammalian signal transduction proteins (Fig. 1c). The *lin-3* gene encodes an epidermal growth factor (EGF)-like molecule that is an inductive signal from the anchor cell²; the *let-23* gene encodes a tyrosine kinase of the EGF receptor subfamily that is a candidate receptor of the inductive signal³; the *let-60* gene encodes a *ras* protein that acts downstream of *let-23* to control vulval cell fates^{4,5}; and the *sem-5* gene encodes a protein with Src homology domains SH2 and SH3 that is likely to act between the *let-23* and *let-60* gene products⁶. Decreased activity of these positively acting genes leads to a failure in vulval differentiation (vulvaless phenotype). Decreased activity of negatively acting genes, *lin-15* and *lin-1*, or hyperactivity of *let-60 ras*, leads to excessive vulval differentiation (multivulva phenotype).

Here we report our genetic and molecular analysis of the *lin-45 raf* gene. This work was initiated by two converging studies: genetic analysis of the *lin-45* gene defined by a mutation that disrupts vulval cell differentiation, and molecular cloning of the *Ce-raf-1* gene, a homologue of the mammalian *raf* family of serine/threonine kinase genes. Our results suggest that *lin-45 raf* gene activity is necessary for vulval differentiation stimulated by the *let-60 ras* gene.

Effect of a *lin-45* mutation

The *lin-45* gene is defined by a recessive mutation (*sy96*) isolated

in a screen for extragenic suppressors of the multivulva phenotype of a *lin-15* mutation⁷. Animals homozygous for the *lin-45(sy96)* mutation display either a larval lethal or a vulvaless phenotype, with or without the *lin-15* mutation in the background. About 91% of the progeny from homozygous *lin-45(sy96)* mothers die as larvae; most survivors are defective in egg-laying (*Egl*⁻) owing to a disruption in vulval differentiation. The extent of vulval differentiation in *lin-45(sy96)* mutants is about 43% at 20 °C compared to 100% in wild-type hermaphrodites (Table 1). Such a vulvaless phenotype suggests that the *lin-45* gene product is involved in specifying normal vulval cell fates.

On the basis of the presence of L1-specific alae and the limited cell division in the gonads of 12 dying *lin-45(sy96)* larvae examined under Nomarski optics^{8,9}, *lin-45(sy96)* larvae die during the first larval stage (L1). Therefore the *lin-45* gene has an essential function during or before the L1 larval stage. L1 lethality has also been observed in animals bearing mutations in other genes in the vulval induction pathway, including *lin-3*, *let-23*, *sem-5* and *let-60* (refs 5-7, 10-13). The homozygous lethality of *lin-45(sy96)* is largely suppressed by wild-type maternal *lin-45* gene activity. The lethality of homozygous mutant animals born from heterozygous (*lin-45/+*) mothers is less than 30%, whereas the lethality of animals born from homozygous (*lin-45/lin-45*) mothers is more than 90% (*N* = 362/396) (Fig. 1b legend). However, this maternal effect does not affect vulval development: about 40% vulval cell differentiation occurs in *lin-45/lin-45* homozygotes born from either *lin-45/+* mothers or *lin-45/lin-45* mothers (Fig. 1b legend). Mutations like *sy96* are relatively rare; the *sy96* allele was isolated after screening ~100,000 mutagenized gametes^{4,11}. The function of the *lin-45*

[†] M.H. and A.G. made equal contribution to this study.

gene in vulval development may be genetically inseparable from its function in early larval viability, such that mutations of the *lin-45* gene that cause a vulvaless phenotype also result in larval lethality.

In addition to the vulvaless and the larval lethal phenotypes, *lin-45(sy96)* mutant animals display other developmental defects. In hermaphrodites, a transformation of P12 cell fate to P11 cell fate in the posterior ventral hypodermis is observed in *lin-45(sy96)* mutants (8 out of 20 animals examined under Nomarski optics). *lin-45(sy96)* males are defective in the development of copulatory structures (H. Chamberlin, personal communication) and thus are unable to mate with hermaphrodites. These defects are similar to those associated with mutations in other genes necessary for vulval induction. Thus, the *lin-45* gene product and the other gene products that regulate vulval induction may be used repeatedly during nematode development.

Cloning of a *C. elegans raf* homologue

We anticipated that additional *C. elegans* gene products would exist in the vulval induction pathway that are homologous to other components of mammalian tyrosine kinase-mediated signalling pathways. The *raf* kinases have been shown to act downstream of receptor tyrosine kinases both in *Drosophila* and in mammalian cell culture. These cytoplasmic serine/threonine kinases mediate *ras*-dependent signalling (for reviews, see refs 14, 15).

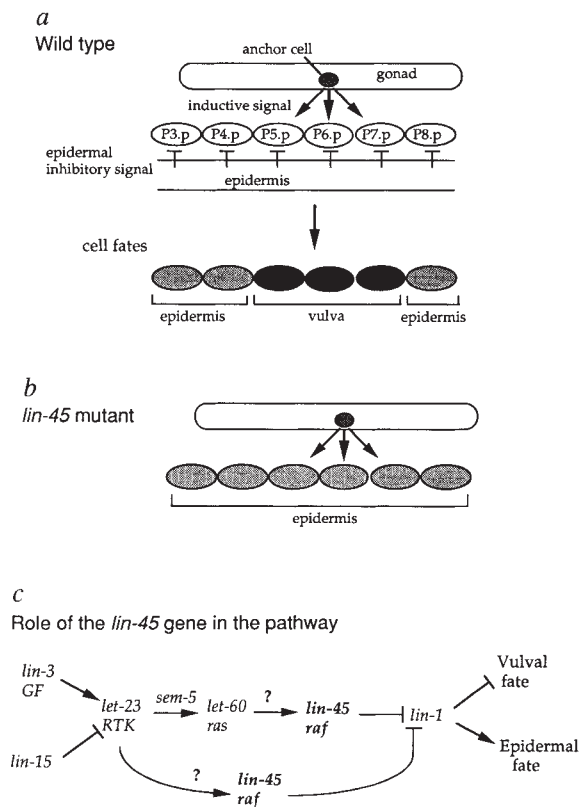
To clone a *C. elegans raf* homologue, we designed degenerate

oligonucleotides based upon two regions highly conserved among the kinase domains of *raf* proteins from various species. With these oligonucleotides, *C. elegans* genomic DNA was enzymatically amplified using the polymerase chain reaction (PCR) (Fig. 2). The PCR-amplified products were used to probe a *C. elegans* complementary DNA library. Seven related clones were identified, the longest of which was sequenced (Fig. 2). This cDNA is likely to be full-length because the extreme 5' end of the cDNA contains sequences homologous to the *C. elegans* trans-spliced leader sequence SL1 (ref. 16). Using this cDNA as a probe, we isolated *C. elegans raf* genomic clones from a yeast artificial chromosome (YAC) library¹⁷ and sequenced a 9.7-kilobase (kb) subcloned genomic fragment.

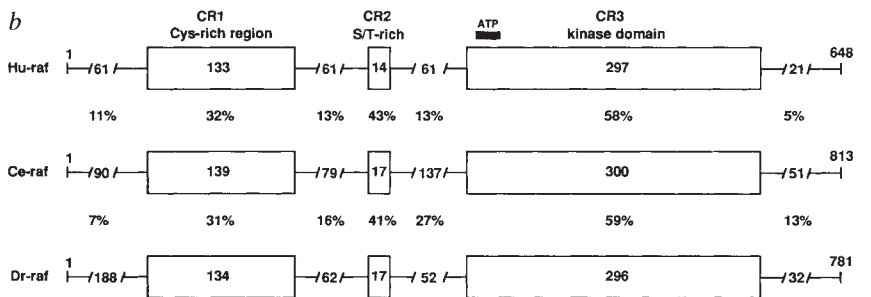
The predicted open reading frame of the cDNA reveals striking amino-acid sequence conservation with the *raf* family of serine/threonine protein kinases, displaying greatest similarity in three regions highly conserved among all *raf* family members¹⁸ (conserved regions 1 (CR1), 2 (CR2) and 3 (CR3); Fig. 3). We therefore designate this gene the *C. elegans raf-1* (*Ce-raf-1*) gene. The *raf* kinases are comprised of two functional domains: an amino-terminal regulatory domain and a carboxyl-terminal catalytic domain¹⁹. The amino-terminal domain contains CR1 and CR2. CR1 is a cysteine-rich, zinc-finger-like domain that, by analogy with a similar domain in protein kinase C²⁰, presumably associates with a regulatory factor(s). This domain has been postulated to inhibit the intrinsic kinase activity of the *raf* proteins; upon binding a factor, inhibition is relieved and the kinase becomes activated. The relative spacing of the

FIG. 1 a, Vulval cell fate specification in wild-type animals. The six pluripotent vulval precursor cells, P3.p–P8.p, are depicted as open ovals. During normal development, an inductive signal from the anchor cell in the gonad induces the three nearby vulval precursor cells (P5.p–P7.p) to adopt vulval cell fates (black ovals)¹. The other three precursor cells (grey ovals) generate part of a large epidermal syncytium. An inhibitory signal, possibly from the epidermis, prevents vulval differentiation in the absence of an inductive signal³⁵. b, An example of vulval cell fates in *lin-45(sy96)* mutant animals. The *lin-45(sy96)* mutation results in less than three of the six vulval precursor cells differentiating into vulval cells (vulvaless). The average vulval cell differentiation in the mutant is about 43% of that observed in wild type (see Table 1). A range of 0% to 100% vulval cell differentiation was observed among *lin-45(sy96)* mutant animals. Vulval differentiation in *lin-45(sy96)* homozygotes from +*lin-45(sy96)* + *unc-24(e138)/unc-44(e362)* + *deb-1(st555)* + heterozygous mothers is 41% (N = 11) (38% for the self progeny of the *lin-45 unc-24* homozygotes, N = 23). *lin-45* heterozygous animals undergo wild-type vulval development. The average number of viable progeny born from *lin-45(sy96)* homozygous mothers is 3.5 (117 progeny/33 mothers). The lethality of *lin-45(sy96)* homozygous animals from *lin-45/+* mothers is greatly reduced: 28% (203/726) of the total progeny of +*lin-45* + *unc-24/unc-44* + *deb-1* + heterozygous mothers were recovered as *lin-45 unc-24* homozygotes. As the *deb-1* allele is embryonic lethal, 33% of the progeny would be expected to be homozygous for *lin-45* and *unc-24*, if there were no lethal effect of the *lin-45(sy96)* allele. c, Proposed functional relationships between the *lin-45 raf* gene and other genes in the *C. elegans* vulval induction pathway. The arrows indicate positive regulation of one gene product by another. 'T' bars indicate negative regulation of one gene product by another. Neither arrows nor 'T' bars necessarily reflect direct interactions. The *let-23* gene product, a receptor tyrosine kinase (RTK)³, is likely to act as a receptor for an inductive signal, the growth factor ('GF') encoded by the *lin-3* gene². The *lin-15* gene is required for the inhibitory signal, which probably acts via the *let-23* RTK (refs 28, 35; L. Huang and P. W. S., unpublished data). The *let-60 ras* gene product acts after the *let-23* RTK but before the *lin-1* gene in the pathway^{4,7}. The *sem-5* gene, which encodes a protein with a SH2 and two SH3 domains, is likely to act between the *let-23* RTK and the *let-60 ras* protein^{6,36}. A decrease in *lin-45 raf* gene activity suppresses the multivulva phenotype caused by a *lin-15(lf)* and a *let-60(gf)* mutation but does not suppress the multivulva phenotype caused by a *lin-1(lf)* mutation (Table 1). Therefore, the *lin-45 raf* gene product may act downstream of the *ras* gene product in the pathway, or it may act in a separate pathway that is required for *ras* function to specify vulval cell fates. If, loss-of-function; gf, gain-of-function.

METHODS. *lin-45 unc-24/+* males were crossed with *unc-44 deb-1/unc-82*



unc-24 hermaphrodites (RW3562; a gift from R. Barstead and R. Waterston). The non-*Unc-24* cross progeny were individually placed on plates and their progeny were examined under a dissecting microscope. The egg-laying ability and the extent of vulval differentiation were examined as described previously^{7,10}. To determine the percentage of the lethal progeny of *lin-45/lin-45* homozygous parents, eggs of rare *Egl⁺ lin-45(sy96)/lin-45(sy96)* hermaphrodites were collected and examined for larval lethality in the following days.



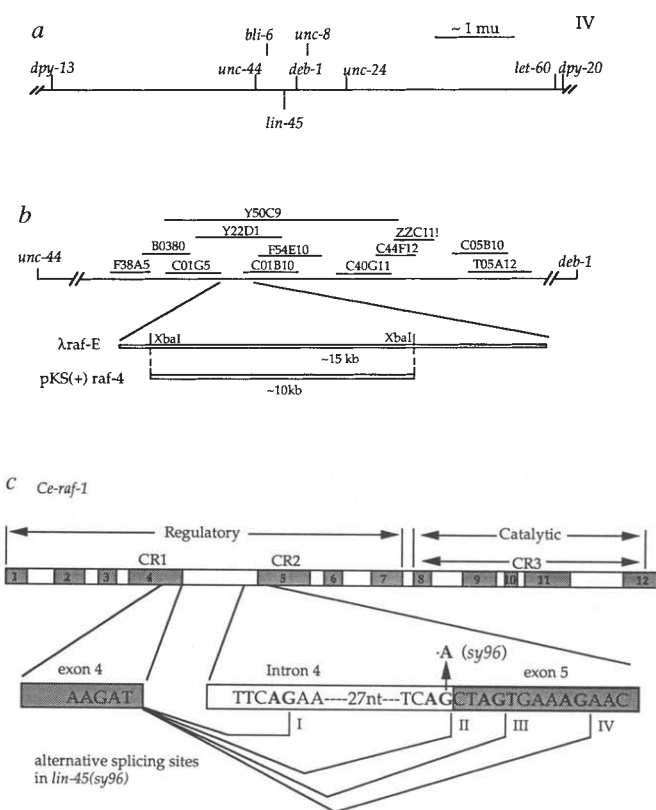
is encoded by the *lin-45* gene, we injected genomic DNA constructs containing the *Ce-raf-1* gene (λ raf-E and pKS(+)*raf-4*; Fig. 4b) into *lin-45(sy96)* mutants. Most homozygous *lin-45(sy96)* animals carrying the injected DNAs as extrachromosomal arrays were egg-laying-competent and produced significantly more progeny on average than *lin-45(sy96)* homozygotes that do not carry the *Ce-raf-1* transgene. Eighty-five per cent of the *lin-45(sy96)* homozygotes bearing injected λ raf-E DNA are egg-laying-competent compared to only 6% of the *lin-45(sy96)* homozygotes bearing only injected marker DNA (Fig. 4b legend). Similarly, phenotypic suppression was also observed when pKS(+)*raf-4* was injected. Therefore, *Ce-raf-1*-containing DNAs suppress the mutant phenotypes of *lin-45(sy96)* animals, suggesting that either the *lin-45* gene encodes the *Ce-raf-1* protein, or high copy numbers of the *Ce-raf-1* gene are able to suppress the *lin-45(sy96)* mutant phenotypes.

FIG. 4 Molecular genetics of the *lin-45* locus. **a**, Genetic map position of the *lin-45* gene. **b**, The physical map around the *Ce-raf-1* gene, illustrated by some representative cosmids and YAC DNA clones (refs 17, 23; A. Coulson *et al.*, personal communication). Two genomic DNA fragments that contain the entire *Ce-raf-1* gene (λ raf-E in a phage vector, and pKS(+)*raf-4*) are also shown. Animals were injected with pRF4 (ref. 44), at a concentration of $75 \text{ ng } \mu\text{l}^{-1}$ and either λ raf-E (at $15 \text{ ng } \mu\text{l}^{-1}$) or pKS(+)*raf-4* (at $8 \text{ ng } \mu\text{l}^{-1}$). Among 176 transgenic animals carrying the injected λ raf-E DNA, 4% of them were egg-laying-defective (Egl⁻), 85% were egg-laying-competent (Egl⁺) and 11% were sterile. Among 152 transgenic animals carrying the injected pKS(+)*raf-4* DNA, 1% of them were Egl⁻, 60% of them were Egl⁺ and 39% were sterile. In contrast, among 32 transgenic animals carrying no exogenous *Ce-raf-1* DNA, 91% of them were Egl⁻, 6% were Egl⁺ and 3% were sterile. The sterile phenotype of some transgenic animals might be due to rescue of the lethal but not of a sterile phenotype by the transgene, or it may be due to an effect of high copy number of the wild-type gene injected. Similar results have been observed in animals carrying *let-60 ras* DNA⁴, suggesting a role for both genes in the production of functional germ cells. The average number of total viable progeny of individual transgenic parents carrying injected λ raf-E, pKS(+)*raf-4*, or only the marker DNA were 44, 53 and 3.5, respectively. These viable progeny include transgenic and non-transgenic animals. **c**, Determination of the molecular lesion of the *lin-45(sy96)* mutant allele. A map of the predicted functional domains of the *Ce-raf-1* kinase is shown above the genomic structure of the *Ce-raf-1* gene. The *C. elegans raf-1* gene has 12 exons (shaded area). CR1 is encoded by exon 4 and CR2 is encoded by exon 5. Part of the DNA sequence of exon 4, intron 4 and exon 5 are shown below the genomic structure. The DNA sequence of the coding regions and the intron/exon boundaries in the *lin-45(sy96)* mutant were determined. The last nucleotide of intron 4 was found to be a G-to-A transition compared to the wild-type *Ce-raf-1* sequence. In the majority of the RNA molecules analysed, the end of exon 4 was spliced to exon 5 at site III, four nucleotides downstream of the site in the wild-type RNAs (site II). But three other alternative splice sites (sites I, II and IV) were also detected at a low frequency (1/45 for each type) in the mutant. One of the sites (site II) corresponds to the position of the wild-type splice site. Other rare alternative splicing patterns may also exist. The majority of the processed RNA in the mutant is not expected to have any normal gene function as RNAs using splice site III are predicted to encode a frameshift mutation in which a stop codon (UGA) immediately follows the novel splice site acceptor. Such RNAs are predicted to encode truncated proteins encoded by only the first four exons, and hence should lack the entire catalytic domain. RNAs utilizing splice sites I and II are predicted to encode protein sequences that stay in-frame and are likely to retain normal function. Protein translated from RNAs utilizing splice site I would encode 11 extra amino acids (Lys-Gln-Phe-Leu-Ser-Lys-Thr-Phe-Asn-Phe-Gln). mu, Map units.

METHODS. We mapped the *lin-45* gene between the *unc-44* and *deb-1* genes on chromosome IV by standard genetic methods³³. From *lin-45/unc-24 dpy-20* heterozygotes, 25 of 25 Dpy non-Unc recombinants carried the *lin-45* mutation. From *lin-45/dpy-13 unc-24* heterozygotes, 3 of 38 Unc non-Dpy recombinants carried the *lin-45* mutation, and 10 of 10 Dpy non-Unc recombinants carried the *lin-45* mutation. From *lin-45/bli-6 unc-24* heterozygotes, 9 of 14 Unc non-Bli recombinants carried the *lin-45* mutation. From *lin-45/unc-44 deb-1* heterozygotes, 8 of 11 Unc non-Deb (Unc non-lethal) recombinants carried the *lin-45* mutation. The references for alleles used are: *unc-24(e138)*, *dpy-13(e184)*, and *unc-44(e362)*³³, *dpy-20(e1282)*³², *bli-6(sc16)* (R. Edgar, cited in ref. 45); *deb-1(st555)* (gift from R. Barstead and R. Waterston). The genomic DNA fragments containing the

lin-45(sy96) disrupts *Ce-raf-1* splicing

To test whether the *lin-45* gene does encode the *Ce-raf-1* gene product, the molecular lesion of the *lin-45(sy96)* allele was determined. Genomic DNA was PCR-amplified with multiple sets of oligonucleotide primers from *lin-45(sy96)* mutant animals as well as from wild-type animals. Sequence analysis of DNA isolated from *lin-45(sy96)* mutant animals revealed a single G-to-A transition in the last nucleotide of the fourth intron (Fig. 4c). As the last two nucleotides (A-G) in the intron are conserved among all *C. elegans* and eukaryotic intron/exon boundaries examined²⁴, the *lin-45(sy96)* mutation might disrupt the normal splicing pattern of *lin-45* RNAs. However, the last conserved G is not absolutely required for correct splicing in *C. elegans*²⁵. To determine the effect of this mutation on the RNA products generated, we sequenced reverse-transcribed RNAs from *lin-45(sy96)* homozygous animals. The majority of *lin-45* transcripts



Ce-raf-1 coding region were obtained by PCR amplification with multiple sets of oligonucleotide primers. DNA sequences of the mutant gene as well as the wild-type gene were determined by directly sequencing the PCR products. To determine the RNA sequence around exons 4 and 5, RNAs were reverse-transcribed with a primer that hybridizes to exon 7 (5'-CGATTAAGCCACCTGGAGGTGATG). The reverse-transcribed product was then PCR-amplified with oligonucleotide primers corresponding to DNA sequences in exon 3 and 7 (5'-TTCGTCCCGGTGAAACCGCGCGGG, and the same oligonucleotide shown above). When the PCR products were run on agarose gels, only one band was detected; it was similar in size to the band detected from wild type DNA. The PCR product was re-amplified with primers corresponding to sequences in exons 4 and 5 (5'-TCAAAGATGCTCATCGTTTGACACC and 5'-CTGTTGCTCATCATTAATCGCAT), and the products were then subcloned into a plasmid vector. The DNA sequences spanning the exon 4 and 5 boundary region were determined for the PCR products by directly sequencing the PCR products as well as by sequencing 45 individual clones. The RNA species that use 3' splice acceptor sites upstream of the original site (within intron 4) were also detected in the mutant by PCR-amplifying the reverse-transcription product. In this experiment, an oligonucleotide that is complementary to the mutant sequence spanning the intron 4/exon 5 boundary region (5'-CTTTCAGTAGTTGAAAATTAATG) was used as the downstream primer. The reaction only detects a band from samples from *lin-45(sy96)* mutant animals and not from wild-type animals.

from the *lin-45(sy96)* homozygotes used a 3' splice acceptor (A-G) four base pairs downstream of the mutated (A-G to A-A) site, creating a frameshift mutant RNA (Fig. 4c; site III). A stop codon immediately follows this splice site. As the predicted catalytic domain of the *Ce-raf-1* protein is located in the carboxyl-terminal half of the gene, the predicted truncated protein resulting from this RNA species is expected to be non-functional. In addition to the major splicing product, three rare splicing sites (one each from a total of 45 clones examined) were also detected among RNAs from the *lin-45(sy96)* mutant (Fig. 4c). Two of these rare splicing products (Fig. 4c; site I and II) are likely to produce functional proteins in *lin-45(sy96)* mutants. These results are consistent with our genetic data and suggest that the *lin-45(sy96)* allele is probably a non-null but severe reduction-of-function mutation. We expect that *lin-*

45(null) alleles would cause recessive larval lethality. As the predicted truncated protein encoded by the predominant RNA (splice site III) contains the region that can potentially bind to an activator^{14,22}, it is possible that such a protein competes for activator binding with the full-length *lin-45* gene products encoded by the other RNA species (splice sites I and II) and thereby further reduces wild-type *lin-45* gene function. But as *lin-45(sy96)/+* heterozygotes and *lin-45(sy96)/lin-45(sy96)/+* animals (*sy96* homozygotes bearing a duplication of the region, *nDp5*) are viable and display wild-type vulval development (data not shown), the vulvaless and the larval lethal phenotypes of the *lin-45(sy96/sy96)* animals are not likely to be due to a toxic effect of a truncated *lin-45(sy96)* protein.

Dominant negative *Ce-raf-1*

To address further the role of the *lin-45 raf* gene in vulval differentiation, we constructed a putative dominant-negative variant of the *Ce-raf-1* gene by mutating the ATP-binding domain²⁶. In mammalian studies, mutational disruption of the consensus sequence G-X-G-X₂-G-X₁₃-K within CR3 (corresponding to residues 488–507 in the *Ce-raf-1* protein; Fig. 3a) has been shown to render the mammalian *raf* kinase inactive²⁷. An overexpressed kinase-inactive variant in mammalian cells is thought to bind and titrate a crucial factor necessary for the activation of the *raf* kinase, thus dominantly interfering with the activation of the endogenous wild-type *raf* kinase^{22,27}. In these systems, expression of such variants inhibits growth factor-stimulated cellular proliferation, *ras*-mediated transformation, and stimulation of downstream gene expression^{22,27}.

We injected wild-type animals with a plasmid bearing a 9.7-kb genomic fragment encoding the *Ce-raf-1* gene product with a single Lys-to-Trp (K-to-W) mutation at position 507. Of the fertile transgenic adults bearing multiple copies of this mutant form of the *Ce-raf-1* gene, approximately 25–45% were egg-laying-defective (Egl⁻). The Egl⁻ defect of the transgenic animals is due to a failure in vulval differentiation (Fig. 5). For one transgenic line, 11 of 28 animals examined did not undergo normal vulval development, suggesting that the vulval precursor cells failed to respond to the inductive signal (9/11 displayed no vulval differentiation and 2/11 displayed partial differentiation). These observations suggest that *Ce-raf-1* gene products containing mutations in the putative ATP-binding domain can effectively interfere with vulval induction. This vulvaless phenotype is similar to the phenotype of animals bearing reduction-of-function mutations of the *lin-3*, *let-23*, *sem-5*, *let-60*, *lin-45*, *lin-2*, *lin-7* and *lin-10* genes of the vulval induction pathway^{5–7,11,28,29}.

Transgenic animals bearing this mutant transgene developed normally except for the vulval defects described. As larval lethality was not observed in these transgenic animals, vulval induction may be more sensitive to the interfering effects of the *raf* dominant-negative variant than the essential function of this gene. A low percentage of transgenic adults were sterile; we have also observed such sterility in transgenic animals bearing the non-mutated *Ce-raf-1* gene.

lin-45 raf probably acts after *let-60 ras*

To determine where in the vulval induction pathway the *lin-45* gene acts, double mutants were constructed with the *lin-45(sy96)* allele and multivulva mutations of the *lin-15*, *let-60* and *lin-1* genes. The extent of vulval cell differentiation of these double mutants was analysed under Nomarski optics (Table 1). The multivulva phenotype of *lin-15(n309)* and *let-60(n1046 gf)* is suppressed by the *lin-45(sy96)* mutation. In contrast, the multivulva phenotype of *lin-1(e1275)* is not suppressed by the *lin-45(sy96)* mutation. As both the *lin-15* and *lin-1* mutations are reduction-of-function mutations¹⁰, the fact that the *lin-45(sy96)* mutation suppresses the *lin-15* and not the *lin-1* phenotypes suggests that the *lin-45* gene probably acts after the *lin-15* gene but before the *lin-1* gene in the genetic regulatory pathway that

TABLE 1 Genetic epistasis

Genotype*	% Vulval differentiation (N)†	Class‡
Wild type	100	Wild type
<i>lin-45</i>	43 (21)	Vulvaless
<i>lin-15</i>	200	Multivulva
<i>lin-45; lin-15</i>	82 (17)	Vulvaless
<i>let-60 (gf)</i>	158 (11)	Multivulva
<i>lin-45 let-60 (gf)</i>	56 (16)	Vulvaless
<i>lin-1 (gf)</i>	165 (13)	Multivulva
<i>lin-1 lin-45</i>	149 (19)	Multivulva

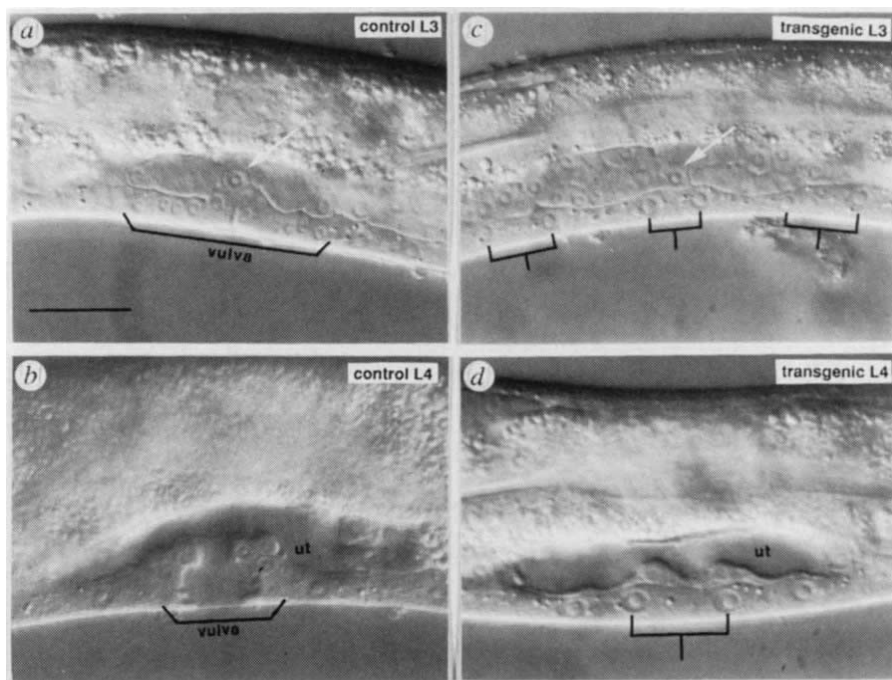
The *lin-45(sy96)* mutation suppresses the multivulva phenotype of *lin-15* and *let-60 (gf)* animals, but not of *lin-1* animals. The *lin-45; lin-15* double mutant was reconstructed after the original *lin-45* allele was outcrossed multiple times. To construct the *lin-45 let-60* double mutant, *lin-45 + dpy-20/+ + +* males were crossed with *let-60(n1046 gf)* hermaphrodites (the *n1046* allele causes an amino-acid change at residue 13 of the protein (Gly to Glu)⁵). The progeny of the F₁ cross that are *lin-45 + dpy-20/+ let-60 +* were screened for non-Dpy Egl⁻ recombinant animals (*lin-45 + dpy-20/lin-45 let-60 +*). The existence of the *let-60* allele in the final double mutant was confirmed by crossing it with *let-60(n1046 gf) +/+ + dpy-20* males. To construct the *lin-1 lin-45* double mutant, *lin-45 unc-24/+ +* males were crossed with *lin-1* hermaphrodites. *lin-1 +/+ + lin-45 unc-24* hermaphrodites were obtained and their progeny with a multivulva phenotype (*lin1/lin-1*) were picked. Several recombinant *Unc* multivulva animals (either *lin-1 lin-45 unc-24* or *lin-1 + unc-24*) were selected from the multivulva parents. They were then tested for existence of the *lin-45* allele by crossing with *lin-45/+* males. The references for the alleles used are: *let-60(n1046 gf)*, and *lin-15(n309)*¹⁰, *dpy-20(e1282)*³², *unc-24(e138)*³³, and *lin-1(e1275)*³⁴.

* The genotypes of the three double mutants are: *lin-45(sy96); lin-15(n309)*, *lin-45(sy96) let-60(n1046 gf)*, and *lin-1(e1275) lin-45(sy96) unc-24(e138)*, where *gf* is gain-of-function.

† Percentage of vulval precursor cells (P3.p-P8.p) that differentiated into vulval cells relative to wild type⁷. The numbers of animals examined under Nomarski optics is indicated (N). In a completely vulvaless animal, each of the six precursor cells divides once and their progeny fuse with the syncytial epidermis (0%). In a wild-type hermaphrodite, three of the six precursor cells divide further than the first round of division, producing the progeny characteristic of vulval cell types (100%) (Fig. 1). Sometimes, only one of the two daughters of a precursor cell divides further to generate vulval tissue; the vulval differentiation in this case is 'one-half cell'. Animals with 100% vulval differentiation do not necessarily have wild-type vulva nor are egg-laying-competent. Among the 21 *lin-45(sy96)* single mutant animals, 7 had 100% vulval differentiation, 8 had 0% vulval differentiation and the rest had between 17 and 67% vulval differentiation. Among the 17 *lin-45; lin-15* animals, 6 had more than 100% vulval differentiation and the rest had between 33 and 100% vulval differentiation. Among the 16 *lin-45 let-60* animals, none had more than 100% vulval differentiation. Among the 19 *lin-1 lin-45* animals, none had less than 100% vulval differentiation (only one had 100%). The data for wild type and *lin-15(n309)* were described previously³¹. The *let-60(n1046 gf)* mutation suppresses the vulvaless phenotype generated by mutations in the *let-23* gene or by ablation of the gonad⁷.

‡ The phenotypes are divided into three classes: wild type (100% vulval differentiation), vulvaless (an average of less than 100% vulval differentiation), and multivulva (an average of more than 100% vulval differentiation).

FIG. 5 Nomarski photomicrographs of transgenic animals bearing a dominant-negative variant of the *Ce-raf-1* gene. Non-transgenic control *dpy-20(e1282)* animals at lethargus of the third larval stage (L3) (a) and at the L4 stage (b). a, Induced vulval precursor cells (VPCs), P5.p, P6.p, and P7.p, have each undergone two rounds of mitosis; a few have entered the third round of mitosis. The bracket marks the progeny of these 3 VPCs. The anchor cell is indicated with a white arrow. The vulva has begun to invaginate dorsally at this stage. b, By the L4 stage, the VPCs have each undergone their final round of mitosis, thus generating all the cells that make up the adult vulva, which is marked by a bracket. The uterine cavity (ut) has formed. c and d, Transgenic animals at L3 lethargus (c) and at the L4 stage (d) bearing the wild-type *dpy-20(+)* gene and the Lys507-to-Trp *Ce-raf-1* variant. The VPCs have undergone only one round of mitosis and their progeny are indicated by lineage trees. In c, the progeny of P5.p, P6.p, P7.p are indicated; in d, only the progeny of the central VPC (P6.p) are indicated. The anchor cell is indicated with a white arrow. There are no signs of vulval invagination, yet uterine development is normal (d). The formation of the uterus is indicative that the animals had reached the L4 stage and that other developmental processes are



not delayed or halted. The VPCs have adopted epidermal fates and have fused with the epidermal syncytium (d). Similar results were observed with two other transgenic lines in which animals displayed incomplete vulval induction (data not shown). Ventral is down in all photomicrographs and anterior is left in a, b and c. Scale bar, 20 microns. Transgenic animals bearing just the marker DNA alone do not display an *Egl*⁻ phenotype. Transgenic animals bearing the non-mutated *Ce-raf-1* gene display a very low penetrance *Egl*⁻ phenotype, far less frequent than that observed for the transgenic animals bearing the mutant transgene.

METHODS. *In vitro* mutagenesis was done by standard protocols⁴⁶ (reagents from Bio-Rad). The mutagenic oligonucleotide, K-to-W: 5'-CGGAAGTGTGC-CATATGGAAGCTGAATGTT-3', substitutes Lys 507 with a Trp residue within the putative ATP-binding site of the *Ce-raf-1* gene product. Incorporated into the mutagenic oligonucleotide is an *NdeI* restriction enzyme site. Incorporation of this restriction site required changing the Ala 505 codon GCU to the synonymous GCC (see Fig. 2). Mutagenesis was done in a sub-cloned *HindIII-SalI* fragment, which was then cloned back into pKS(+)-raf-

4. Plasmids bearing the K507-to-W mutation were injected into homozygous recessive *dpy-20(e1282)* animals. Injections were done as described⁴¹, using pMH86 as a selectable marker (ref. 47; D. Clark and D. Baillie, personal communication). The mutant *Ce-raf-1* DNA was injected at a concentration of 200 ng μl^{-1} with pMH86 at a concentration of 10 ng μl^{-1} . Approximately 10% of the F₁ transgenic animals stably transmitted the transgenic arrays to their progeny. Because the arrays exist extrachromosomally and can be lost mitotically during development, only animals that were non-Dpy through the L4 stage were used for this analysis. The incomplete penetrance of the *Egl*⁻ phenotype may reflect the fact that many of the transgenic animals are mosaic because of mitotic loss of the transgenic array. These numbers may also reflect the possibility that higher doses of expression of the mutant DNAs are needed for complete disruption of vulval induction. It also remains possible that other pathways operate in the absence of endogenous *raf* activity to induce vulval differentiation. For photographs, transgenic animals were anaesthetized in sodium azide⁴⁸ and staged by standard criteria^{8,9}.

specifies vulval cell fates. The *let-60(n1046 gf)* mutation (Gly13 to Glu) results in constitutively high *let-60 ras* gene activity and a semi-dominant multivulva phenotype⁵. Suppression of the multivulva phenotype of the activated *let-60 ras* mutation by the *lin-45(sy96)* mutation suggests that the *lin-45 raf* protein acts after the *let-60 ras* protein in this signal transduction pathway. It is also possible that *lin-45* and *let-60* act in separate pathways that are both required for vulval cell differentiation (Fig. 1c).

Our genetic experiments, which order the activities of the *let-60 ras* and *lin-45 raf* genes during *C. elegans* vulval induction, complement the findings observed in mammalian and *Drosophila* studies. Specifically, the *raf* gene product appears to act downstream of the *ras* gene products in mammalian growth-factor-mediated signalling^{14,15}, in the *Drosophila* R7 photoreceptor cell induction pathway³⁰, and in the vulval induction pathway of *C. elegans*. All of these signalling pathways are stimulated by inductive signals whose receptors are tyrosine kinases. Together, the data from these three systems suggests that *raf* serine/threonine kinases act downstream of or in parallel to *ras* proteins to mediate intracellular responses to various external stimuli. A tyrosine kinase-*ras-raf* signalling pathway might constitute a signalling cassette commonly used in proliferative and developmental programs of a variety of metazoa. □

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Evidence that the $z = 3.4$ radio galaxy B2 0902 + 34 may be a protogalaxy

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THE monochromatic luminosities of high-redshift ($z > 3$) radio galaxies rise steeply between wavelengths of about 2,000 and 5,000 Å, to form a characteristic 'red bump'^{1–6}. It is usually assumed that this bump arises from the photospheric emission of red, post-main-sequence stars. For a sufficient number of stars of this type to have evolved, however, these galaxies must be at least 0.4–2 Gyr old; yet $z = 3$ corresponds to only 1.7 Gyr after the Big Bang (assuming a Hubble constant of $50 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and that $\Omega_0 = 1$), bringing the larger age estimates uncomfortably close to the beginning of the Universe. Here we show that, at least in the case of the high-redshift radio galaxy B2 0902 + 34, the basic assumption is incorrect: the red bump is caused not by photospheric emission from post-main-sequence stars, but by the presence of bright emission lines from doubly ionized oxygen. Both the spectrum and the luminosity of the underlying continuum suggest that B2 0902 + 34 is a galaxy observed during its initial burst of star formation.

The conclusion that high-redshift radio galaxies contain old stars is based on spectral energy distributions (SED) constructed from fluxes measured through broad-band filters, and these necessarily have low spectral resolution ($\lambda/\delta\lambda \approx 5$ –10). Observations of high-redshift radio galaxies with higher spectral resolution are valuable because of the possibility of observing spectral features that are better measures of age than the overall shape of the continuum^{7,8} and because the low-resolution SEDs may be distorted by the presence of emission lines in the broad-band filters. The SEDs of radio galaxies at $z > 3$ are particularly likely to be affected by this second possibility because of a doublet of emission lines from doubly ionized oxygen ([O III] at 495.9 and 500.7 nm) which at these redshifts appears in the near-infrared (2.2 μm) K-band.

B2 0902 + 34 was the first radio galaxy with a very high redshift ($z = 3.4$) to be discovered and was originally believed to have an extremely red optical–infrared colour, implying an age of ~ 1 –2 Gyr (ref. 1). To investigate the effect of the oxygen doublet on the SED, we observed this galaxy with the new CGS4 spectrometer on the United Kingdom Infrared Telescope (UKIRT). Figure 1 shows the reduced spectrum which has a

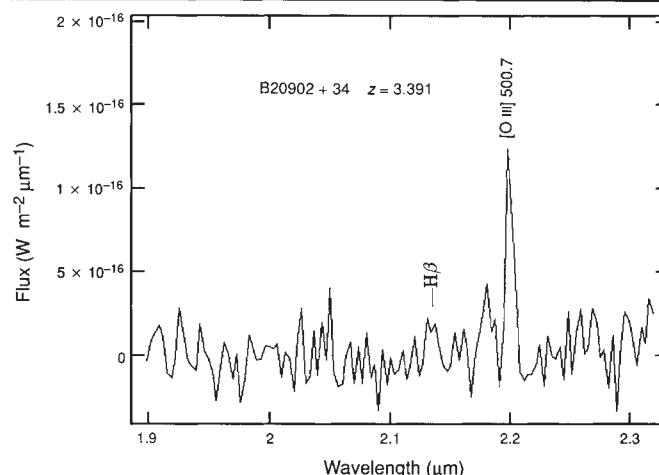


FIG. 1 Spectrum of B2 0902 + 34. We obtained this spectrum on the night of 1992 January 12 using the CGS4 spectrometer on the UKIRT. The position we used for the galaxy was the radio position given in ref. 1. The galaxy was observed with the slit at position angle 85° east of north, which is the position angle of the optical structure of the galaxy¹. The slit had a width of 3.1 arcsec and the spectrum is for a square aperture with a side of length 3.1 arcsec and at a position angle of 85° east of north. Further details of the instrument and of the observing procedure are given in ref. 26. The fluxes of the [O III] 500.7 and the $H\beta$ lines are $1.04 \pm 0.19 \times 10^{-18} \text{ W m}^{-2}$ and $0.37 \pm 0.18 \times 10^{-18} \text{ W m}^{-2}$, respectively. The [O III] 495.9 line is always precisely 3 times fainter than the [O III] 500.7 line²⁷. The total flux in emission lines, assuming this ratio, is $1.75 \pm 0.33 \times 10^{-18} \text{ W m}^{-2}$.

spectral resolution of ~ 340 and covers the entire K-band. The [O III] lines at 495.9 and 500.7 nm and the $H\beta$ line were detected, although the detection of the $H\beta$ line was only marginally significant (2σ). The presence of bright emission lines and the absence of any obvious continuum emission raises the question of how much of the apparent red bump in the low-resolution SED of this galaxy is actually caused by emission lines rather than old stars.

To determine quantitatively the effect of the emission lines on the SED, it is necessary to compare the flux in the emission lines with the flux measured from a K-band image. As the infrared image in the discovery paper is known to be incorrect^{1,9,10}, and as there were no other published infrared images, we obtained a new image of this galaxy with the infrared camera (IRCAM) on the UKIRT. Figure 2 shows our image. We estimated a K-band magnitude for the continuum emission by measuring both the line flux and the broad-band flux through exactly the same aperture (calibrating them from standard star fluxes measured through the same aperture) and then subtracting the line flux from the broad-band flux using the overall transmission function for the K-band (transmission function of the filter times the transmission function of the atmosphere). Our K-band magnitude, uncorrected for lines, measured through a circular aperture of diameter 4 arcsec ($K = 19.7 \pm 0.2$) is consistent with a contemporaneous measurement¹¹.

Figure 3 shows the SED for the galaxy after subtracting the line emission from the K-band flux; also shown are points corresponding to the uncorrected K-band magnitude and to the

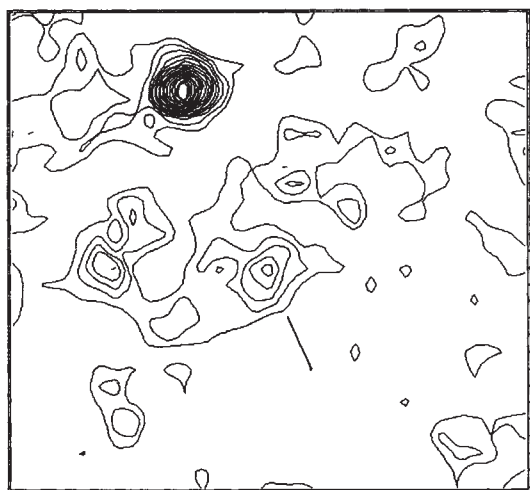


FIG. 2 K-band image of B2 0902+34. The image is a square of side 40 arcsec. North is at the top and east is to the left. The contours are at $\sim 1\sigma$, 2σ , 3σ ..., where σ is the noise on the image. An arrow shows the position of the radio galaxy. Many of the extremely faint objects visible on the image are also present on the deep optical image¹. We obtained the infrared image using the infrared camera (IRCAM) on the UKIRT in its 1.24 arcsec per pixel mode. As part of the UKIRT service observing program, B2 0902+34 was observed for 54 minutes on the night of 1992 February 19 and for 60 minutes on the night of 1992 March 15. The standard observing procedure of making a mosaic of sky-limited observations about the position of the radio galaxy was followed, as this makes it possible to construct a flat-field out of the data themselves²⁸. The large plate scale made it possible to use a large offset in the mosaicing of 16 arcsec, which proved a substantial benefit because of the complicated structure in the field. Both bright and faint standard stars were observed on each night, the faint standard stars being observed to check that the array had a linear response. The data were reduced in the standard way²⁸, and the images obtained on the two nights were added together to produce the final image, using the bright object to the north east of the radio galaxy for astrometric registration.

erroneous magnitude given in the discovery paper¹. We see that the original measurement led to an overestimate of the height of the red bump, but that a substantial red bump is present even using the correct broad-band value. If, however, we consider the continuum emission left after allowing for emission-line contamination, we see that although we cannot rule out the presence of some post-main-sequence stars, there is no longer any evidence for this. The SED predicted for a galaxy in which there are no post-main-sequence stars is flat between the wavelength of Ly- α (1,216 Å) and $\sim 5,000$ Å (ref. 12). The line-corrected SED of B2 0902+34 is clearly consistent with this, in contrast to the uncorrected SEDs of all other high-redshift radio galaxies of which we are aware. Eisenhardt and Dickinson¹¹ have obtained an image of this object taken through a narrow-band filter centred on the [O III] doublet. They, too, concluded that the emission in the doublet is high, but they were not able to determine whether the contribution of the emission lines could explain the red bump, because they did not have a sufficiently sensitive K-band image.

The other piece of evidence that this object is a young galaxy is its low optical luminosity. The absolute V-band ($0.5 \mu\text{m}$) magnitude of the continuum emission of B2 0902+34 can be straightforwardly calculated from the K-band magnitude (after subtracting the contribution of the emission lines), because of the close correspondence between the K-band on Earth and the V-band in the rest-frame of the galaxy. We find $M_V = -21.4^{+0.2}_{-2.2}$ (90% confidence limits). This is 1.5 magnitudes fainter than the radio galaxies from the 3C catalogue at $z \approx 1$, which have similar radio luminosities and optical structures to the radio galaxies known at $z > 3$ (ref. 13). If the idea that radio galaxies formed at very early times^{1,2} is correct, B2 0902+34 should actually be

about 2 magnitudes brighter than the lower-redshift radio galaxies as a result of the gradual evolution of the main-sequence turn-off mass, 'passive' stellar evolution¹⁴⁻¹⁶. It is particularly interesting to compare B2 0902+34 with the $z = 1.82$ radio galaxy 3C326.1, as this was once thought to be a protogalaxy¹⁷. More recently it has been argued that 3C326.1 is a normal radio galaxy, with its faint K magnitude just being a 2σ deviation, which will occur 5% of the time for any population whose luminosity has a standard deviation of σ (ref. 18). The absolute V-band magnitude of B2 0902+34, however, is an additional 2 magnitudes fainter than that of 3C326.1, making it difficult to extend the argument of a statistical fluctuation to B2 0902+34, especially as there are few radio galaxies known at these high redshifts. The natural explanation of the low optical luminosity of B2 0902+34 is that this object is at the beginning of its initial burst of star formation, when only a fraction of the stars that will constitute the galaxy have formed. The bright [O III] emission lines prove that at least one generation of stars has formed in the galaxy, but this provides only a weak limit (10^7 years) on galaxy age which is much less than the minimum dynamical timescale for galaxy formation¹⁹.

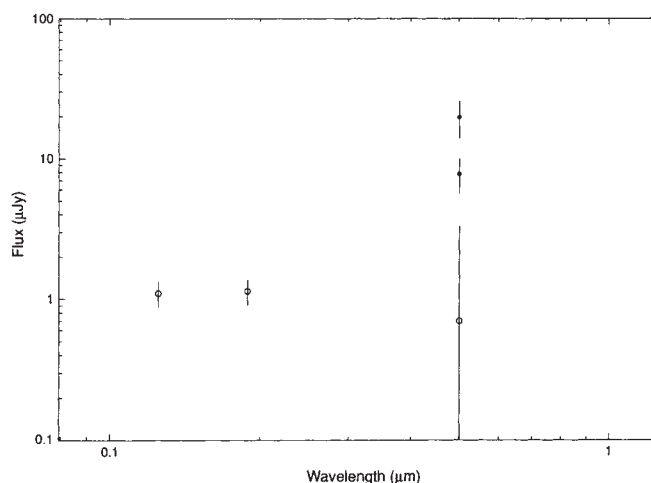


FIG. 3 Spectral energy distribution of B2 0902+34. The optical data are from ref. 1. The fluxes are plotted at their wavelengths of emission rather than observation. There are three K-band flux densities plotted: the flux from the discovery paper¹ (high); our flux without correcting for the lines (middle); our flux after the lines have been subtracted (low). Our uncorrected flux, measured through the same 3.1×3.1 arcsec² square aperture as was used for the spectrophotometry, is equivalent to a K-band magnitude of $19.8^{+0.4}_{-0.3}$; our corrected K-band flux is equivalent to a K-band magnitude of $22.4^{+0.0}_{-1.7}$.

There are two alternative explanations which should be considered. One possibility is that the characteristic red SED of the underlying galaxy is being concealed either by light produced by young stars formed in a burst some time after the formation of the galaxy or by scattered light from the active nucleus, a process which has been seen in radio galaxies at lower redshifts²⁰⁻²⁴. Although this could explain the blue SED, it would make the difference between the optical luminosities of the original stellar population and of the $z \approx 1$ radio galaxies even greater. The second possibility is that the low optical luminosity of B2 0902+34 could be increased by using a non-zero cosmological constant (by 1.5 magnitudes relative to the radio galaxies at $z \approx 1$ for a spatially flat universe with $\Omega_0 = 0.2$ and $\lambda = 0.8$), an option which has been suggested as a solution to other cosmological problems²⁵. This makes the optical luminosities of other radio galaxies at $z \approx 3$ hard to explain by stellar evolution—they can be as much as 5 magnitudes brighter

than B2 0902 + 34 (ref. 26)—but this could be avoided if there is a nonstellar component to the light from the more luminous systems (ref. 26).

Thus, unless the cosmological constant is not zero, we think it likely that B2 0902 + 34 is a young elliptical galaxy. This raises the question of whether B2 0902 + 34 is unique or whether there are other young high-redshift radio galaxies. There is now little evidence that any radio galaxy at $z > 1$ is old. First, we have shown here that the red SEDs of the highest redshift radio galaxies can prove unreliable evidence for an evolved stellar population. Second, there is now evidence that at least some of the optical light from high-redshift radio galaxies is scattered nonstellar light^{20–24}. Third, if these galaxies are old galaxies one would expect a sharp rise in the optical spectrum at 4,000 Å, and recent spectroscopic observations of radio galaxies at $z \approx 1$ have found no sign of this⁸. The chief remaining evidence for these objects being old is the small scatter in the infrared Hubble diagram at $1 < z < 2$ (ref. 2), which breaks down at $z > 2$ (ref. 6), an effect due only in part to line contamination²⁶. We suspect that re-examination of the tightness of this relation as a function of redshift and radio luminosity will be the best way of determining exactly when giant ellipticals formed and the role of radio emission in this process. □

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Reconciling the magnetic field structures seen in variable active galactic nuclei with the unified scheme

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IN the orientation-based unification scheme for active galaxies, the most variable active galactic nuclei—the BL Lacertae objects and highly polarized quasars (HPQs)—correspond to radio galaxies with their relativistic jets oriented close to our line of sight^{1–7}. This otherwise successful scheme has recently been challenged by radio polarimetric very-long-baseline interferometry^{8,9}, which reveals different magnetic field structures in the knots of synchrotron emission identified with shocks in these jets. For BL Lacs the field is generally perpendicular to the jet, whereas no such trend is apparent for HPQs. This dichotomy has led to the claim that luminous BL Lacs and HPQs are inherently different types of object¹⁰. Here we propose that this difference can be readily explained within the unified scheme by the effect of relativistic aberration: at small viewing angles, the observed knot emission arises primarily from the freshly excited plasma of the shock front, whereas at larger angles, Doppler-boosted emission from the slower, post-shock plasma is the dominant contribution. We identify the former case with BL Lacs and the latter with HPQs.

Many observations of radio-loud active galactic nuclei (AGN), including radio flux and polarization monitoring data for BL Lacs and HPQs, are currently best interpreted in terms of shocks propagating down relativistic flows in jets^{11,12}, giving rise to outbursts and knots of synchrotron emission whose positional changes with time can be followed by very-long-baseline interferometry (VLBI)^{13,14}. The variability of BL Lacs is explained by postulating that the jet alignment is so close to

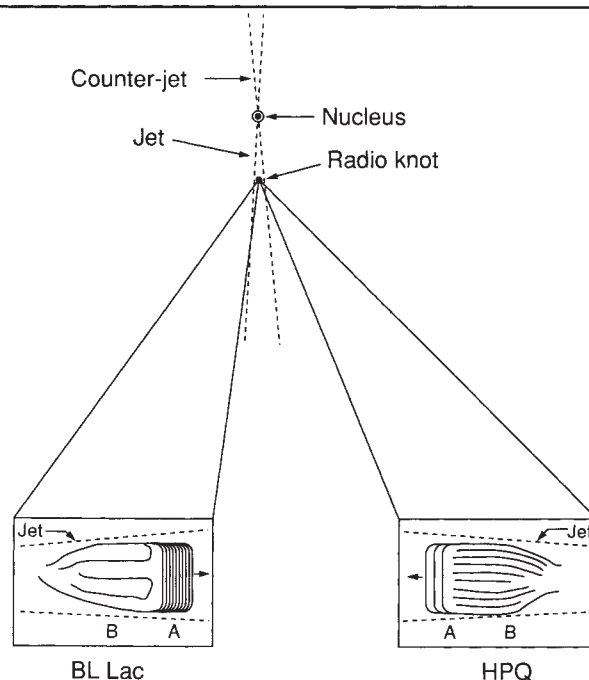


FIG. 1 Schematic representation of the predicted apparent fine structure of the parsec-scale (essentially unresolved) radio knots for BL Lacs and HPQs, as proposed in our model. The orientations of the lines represent the direction of the magnetic fields in the regions A and B of the knots, although the depicted complete realignment in region B is a gross idealization, and their spatial concentrations represent the observed flux densities of the two regions, which are of intrinsically similar strengths. The different microstructures of the moving radio knots for BL Lacs and HPQs arise from the closer alignment of the BL Lac jet to the line of sight and from the somewhat smaller Lorentz factors for the relativistic plasma in region B.

the line of sight ($\theta < 10^\circ$) that the Doppler-boosted synchrotron continuum overwhelms all other emission components¹. Although the details of the shock structure and magnetic field configuration in relativistic flows are still uncertain, some aspects

are generally adopted in models. For instance, the radio emission is believed to arise from relativistic charged particles gyrating in a magnetic field (synchrotron radiation), so that the observed linear polarization vector is perpendicular to the average magnetic field direction in the source. It is also usually supposed that the peak of fractional polarization coincides with the shock front, although the limited resolution of VLBI cannot exclude the possibility that it sometimes corresponds to dimmer regions offset from the shock¹⁵. Here we show that such general considerations suffice to resolve the apparent contradiction between the VLBI polarization maps of powerful BL Lacs and those of HPQs.

In our simplified picture, the region disturbed by the shock consists of two regimes: (A) material just compressed by the outward moving shock, and (B) material further behind the shock front, with a diminished bulk Lorentz factor (Fig. 1). In the quiescent jet the magnetic field is expected to be predominantly aligned along the jet flow, with a small random component^{16,17}. Because of the shock compression, the component of the random magnetic field perpendicular to the jet is amplified in region A by a factor depending on the strength of the shock, such that for moderately strong shocks, in the densest region just behind the shock where synchrotron emissivity is steeply enhanced¹⁸, it dominates over the parallel component. For a nonrelativistic jet, the maximum enhancement of polarization will thus be observed when viewing the jet perpendicular to its flow direction, but for a relativistic jet containing a shock moving with velocity v , this edge-on view of the shock occurs for the critical angle $\theta_c = \sin^{-1}(1/\Gamma)$, where $\beta = v/c$ and $\Gamma = (1 - \beta^2)^{-1/2}$ is the bulk Lorentz factor for the shock¹⁶⁻¹⁸. This condition also maximizes the apparent proper motion of the VLBI knot associated with the shock, which would thus show a strong polarization along the jet¹⁸.

Radiation emitted in the plane of the shock whose normal makes an angle θ to the line of sight would appear¹⁹, because of relativistic aberration, to arrive at the observer at an angle $\theta = \cos^{-1}[(\cos \theta - \beta)/(1 - \beta \cos \theta)]$. The fractional polarization of such an emission region has the following dependence^{12,18} on the angle θ' : $\Pi = (1 - \alpha)[(1 - \kappa^2) \sin^2 \theta'] \times \{(\frac{2}{3} - \alpha)[2 - (1 - \kappa^2) \sin^2 \theta']\}^{-1}$ where κ is the factor by which the jet plasma is compressed at the shock, and α is the spectral index, defined by relating the flux density to the frequency: $S_\nu \propto \nu^{-\alpha}$. Also, the intensity of emission from any moving knot would appear Doppler-boosted by the factor $\delta^{3-\alpha}$, where the Doppler factor $\delta = [\Gamma(1 - \beta \cos \theta)]^{-1}$ (refs 1, 2).

The material further behind the shock (region B) gradually expands and merges with the ambient jet flow; in this process the shear is likely to lead to a partial realignment of the magnetic field towards its original mainly parallel orientation^{17,20}. Thus, the polarization angle of the synchrotron emission from region B will vary, as its magnetic field will have a helical structure. Because during this phase the post-shock fluid remains denser than the ambient jet plasma, and its magnetic field is still fairly high, its synchrotron emissivity is higher than for the undisturbed jet. Also, this recovering plasma continues to propagate towards the observer, but with a reduced bulk Lorentz factor, so the beaming cone for this material will be wider than for the freshly shocked material in region A.

Region A, with the highest compression and strongest fields, and therefore the highest emissivity, will be relatively small. Region B, with lower density and weaker field, will have lower emissivity, but as it is larger the synchrotron output should be comparable to that of A. The polarization vectors of these two components will be substantially misaligned. As discussed above, their relative contributions to the observed total and polarized intensities will largely depend on their Doppler factors, $\delta_{A,B}$.

In conformity with the unified model, we propose that the jets observed at small viewing angles ($\theta < \theta_{c,A}$) are classified as BL Lac objects. In this case, the emission from region A will

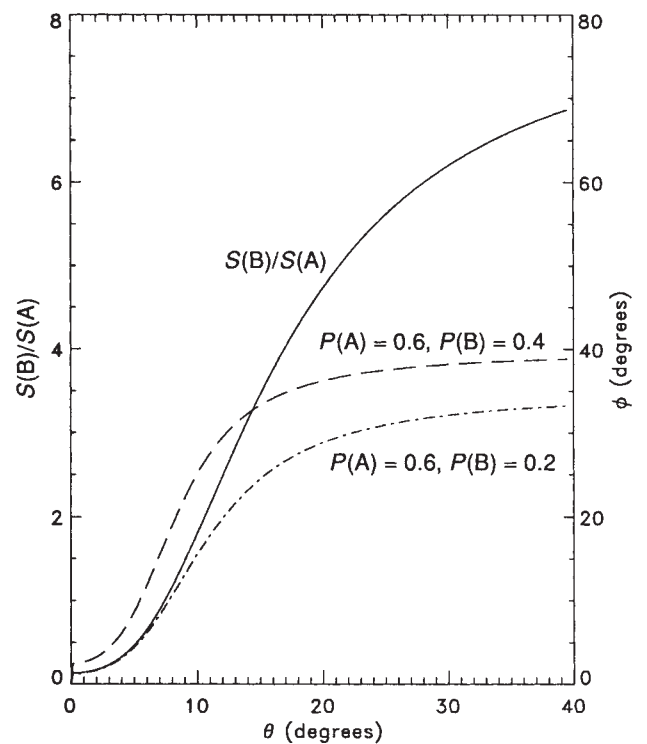


FIG. 2 Relative observed intensities $S(A)$ and $S(B)$ of regions A and B and the polarization angle of their combined synchrotron emission (measured from the jet axis, so that $\phi=0$ implies a magnetic field perpendicular to the jet), computed for a reasonable and typical case of our model, are plotted against the angle between the jet flow and the observer's line of sight, θ . For simplicity we assume equal flux densities for regions A and B in their rest frames, and that they both have $\alpha = -1$; here $\Gamma_A = 10$ and $\Gamma_B = 6$. The two curves illustrating the substantial expected swings in the net polarization vector are labelled with the adopted high fractional polarizations $P(A)$ and $P(B)$ of components A and B, as indicated by VLBI observations (ref. 9). Here we have assumed that the magnetic field orientations differ by 45° between components A and B, and even larger changes could arise for bigger misalignments. For $\theta > 10^\circ$, component B dominates the flux, and only for $\theta < 10^\circ$ is $\phi < 20^\circ$, characteristic of BL Lac knot polarization.

dominate, explaining the observed strong alignment of polarization vectors with the jet direction^{10,11}. But at larger viewing angles, relativistic aberration permits a direct view of region B behind the shock²¹, even at frequencies where the shocked region A might be opaque. Simultaneously, the flux density of region B relative to that of A increases as its lower Lorentz factor creates a wider cone of Doppler-boosted emission (Fig. 2). Thus for sources viewed at moderate angles ($\theta_{c,A} < \theta < \theta_{c,B}$) the measured polarization should mainly reflect the magnetic field orientation in region B, which is expected to depart substantially from the plane of the shock. Within our simple unified picture, such objects would be identified as HPQs and objects viewed at still larger angles as radio-loud quasars, whereas those seen at $\theta > 45^\circ$ would appear as powerful (FR II) radio galaxies⁴. The strong tendency for the HPQs to have polarization vectors grossly misaligned with the direction of jet flow can thus be qualitatively understood. It also seems plausible in this picture that polarization even of bright VLBI knots may sometimes be low if the viewing angle is such that the polarized components in regions A and B largely cancel each other; such variations in viewing angle could temporarily arise even within the same source from a wiggling jet^{17,22}. Examples of such cases can be found in the published VLBI maps⁹.

Over the past few years evidence has been mounting that BL Lac objects have two parent populations²³⁻²⁵. In both types the synchrotron emission is enhanced by orientation effects, so it is understandable that they were mistakenly grouped together. The

existence of two parent populations is supported by the discovery of two peaks in their redshift distribution (near 0 and near 1),²³ and by their variability over a much wider range than HPQs (which all show large radio variability)²⁴. The nearby, lower-luminosity BL Lacs are probably the less-luminous FR I radio galaxies with jets viewed close to the line of sight^{23,26,27}, and having lower bulk Lorentz factors than jets in the more powerful distant BL Lacs whose parent population is likely to be FR II radio galaxies^{4,25}. X-ray-selected samples preferentially contain nearby (redshift $z < 0.3$) BL Lacs with Lorentz factors modest compared with $\Gamma \approx 10$ for radio-selected BL Lacs, which frequently lie at greater redshifts²⁷. The magnetic field orientations of the VLBI knots are similar in the two groups of BL Lacs⁹. This homogeneity is readily understood in our picture because the emission from region A in the weaker BL Lacs would continue to dominate up to fairly large viewing angles, owing to their smaller Lorentz factors.

A second systematic difference between powerful BL Lacs and HPQs is that the radio core components of the former are more highly polarized than those of the latter⁹, although in both classes the knots frequently show higher degrees of fractional polarization. The reduced polarization of the cores could arise from Faraday effects⁹ caused by the magnetoionic plasma associated with filaments of thermal gas in the broad line region immediately surrounding the core. Obscuration by such filaments is expected to be less severe for the BL Lac cores, because, in our model, they are being viewed almost directly through the jet channel, from which most thermal material has presumably been dispersed. Nonetheless, a reduction in the fractional polarization, and a concomitant rotation of the polarization vector, can be expected even for some BL Lac cores, in agreement with the VLBI observations⁹.

In our model, the mean of the Lorentz factors for powerful BL Lacs should slightly exceed that for HPQs. Recently, the synchrotron-self-Compton model has been applied²⁸ in conjunction with the measured sizes and proper motions of the VLBI knots to estimate the Lorentz factors for different types of AGN. For 16 powerful BL Lacs (with $z > 0.2$) the average minimum Lorentz factor is estimated to be $\langle \Gamma \rangle = 3.6 \pm 2.0$, whereas for a set of 24 HPQs the corresponding value is estimated to be 6.0 ± 3.0 (ref. 28). This apparent marginal excess of Γ for the HPQs is likely to be an artefact of the assumption that the radio-emitting volumes are spherical. In our picture, the radio knots in BL Lacs are disk-shaped, and only their major axes are likely to be resolved by VLBI, whereas for HPQs the spherical approximation may be realistic (see Fig. 1). The true volumes for the BL Lac knots could easily be several times smaller than those estimated; because $\delta \propto \text{volume}^{-0.56}$ for a typical spectral index of -1.0 (ref. 28), δ (and thus Γ) could be severely underestimated. Thus the current data are not inconsistent with our hypothesis requiring BL Lacs to have somewhat higher Lorentz factors than HPQs.

The VLBI data on complete samples demand a range of Γ factors¹³, but in a flux-limited sample the characteristic value will be closer to the peak ($\Gamma \approx 10$). Such large values of Γ associated with BL Lacs can be reconciled in our picture with the fact that their observed superluminal speeds ($\beta_{\text{app}} < 4$) are smaller than the typical values of 8–10 for HPQs⁷, because BL Lacs are being viewed at small angles $\theta < \theta_{c,A}$ (refs 1, 5). A few powerful BL Lacs may have their observed continuum emission enhanced through gravitational microlensing by stars in an intervening galaxy^{29,30}. But for most sources it appears that some striking new observational results on AGN can be successfully addressed within the framework of the orientation-based unified scheme. $\square$

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Mechanism for the acceleration and ejection of dust grains from Jupiter's magnetosphere

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PERHAPS the most unexpected finding of the Ulysses mission so far has been the detection of quasi-periodic streams of high-velocity, submicrometre-sized dust particles during the spacecraft's encounter with Jupiter¹. The impact geometry clearly shows that these small grains originate in the jovian system, but it is surprising that any dust can escape Jupiter's gravitational influence. Here we show how the Ulysses dust events could result from the acceleration and subsequent ejection of small grains by Jupiter's magnetosphere. Dust grains entering the plasma environment of the magnetosphere become charged, with the result that their motion is then determined by both electromagnetic and gravitational forces. We have modelled this process and find that only those particles in a certain size range gain sufficient energy to escape the jovian system. Moreover, if Io is assumed to be the source of the dust grains, its location in geographic and geomagnetic coordinates determines the exit direction of the escaping particles, providing a possible explanation for the observed periodicities. The calculated mass and velocity ranges of the escaping dust grains are consistent with the Ulysses findings.

Let us first summarize the observations. The mass of detected stream particles is $1.6 \times 10^{-16} < m < 1.1 \times 10^{-14}$ g and their velocity is $20 < v < 56$ km s⁻¹. Assuming an average density of 1 g cm⁻³, the size range of these particles is $0.03 < a < 0.14$ μ m. The streams were spaced $\sim 500 R_J$ apart ($R_J = 7.1 \times 10^4$ km, the radius of Jupiter) and they seem to arrive in bursts with a period of 28 ± 3 days. These grains originate from within the jovian system, suggesting that their source is the dusty regions of the jovian magnetosphere or the volcanoes on Io. As Io offers the

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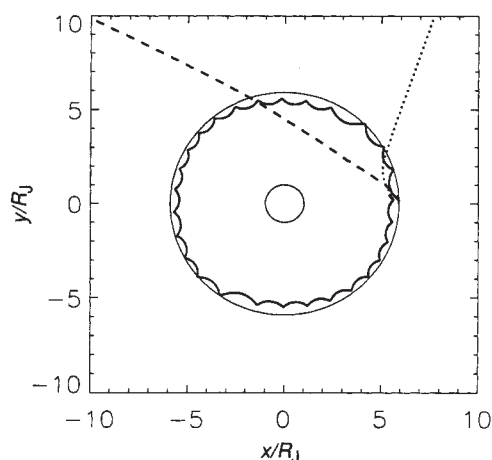


FIG. 1 The trajectories of dust particles with sizes $a=0.02$ (solid line), 0.03 (dotted line) and $0.1 \mu\text{m}$ (dashed line) started from Io. At $t=0$ the longitude of Io is zero in both the inertial frame and in magnetic coordinates. The two circles represent the surface of Jupiter and Io's orbit (thin solid lines).

most natural way to explain the observed periodicity, we show for this source how ejection of small grains might occur.

Io is a likely source of small dust grains in the Jovian magnetosphere². Small grains entrained in volcanic plumes follow ballistic trajectories and reach altitudes where they become exposed to the Io plasma torus. As grains encounter this high-density plasma region, they rapidly collect electrostatic charge and interact with the local magnetic fields. For grains smaller than $0.1 \mu\text{m}$, the resulting Lorentz force overcomes Io's gravity and these grains become injected into the jovian magnetosphere. The injection velocity is $\sim 57 \text{ km s}^{-1}$, the relative velocity between the co-rotating magnetic field and Io^{2,3}. This production process sets an upper limit on the size of the dust particles escaping Io. To determine the charge of dust grains in the magnetosphere we used a detailed model describing the composition, density and temperature of the jovian plasma environment⁴. The potential of grains was calculated considering all the important charging currents: the thermal fluxes of electrons and ions, photoelectron emission and secondary electron emission. In general these charging currents are modified by the

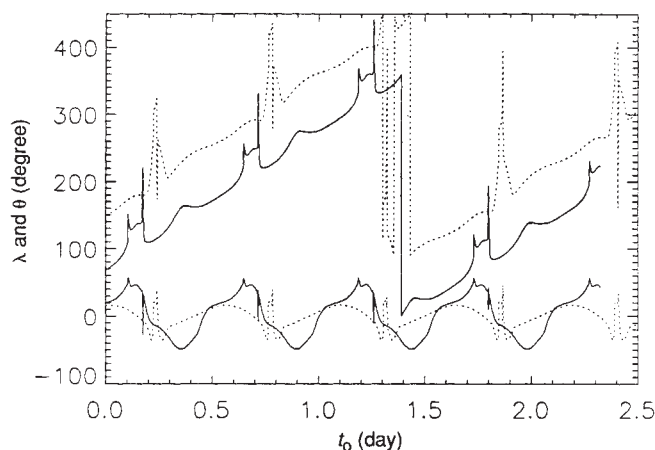


FIG. 2 The longitude, λ (upper curves) and the latitude, θ (bottom curves) of particles with $a=0.03$ (solid lines) and $0.1 \mu\text{m}$ (dashed lines) on exit from the jovian magnetosphere (at $r=50R_J$) as function of the time (t_0) at which they were released from Io. At $t_0=0$ Io's longitude is zero in both the reference frame and in magnetic coordinates. The characteristic transit time from 6 to $50R_J$ is ~ 0.6 and 1.1 days for particle sizes $a=0.03$ and $0.1 \mu\text{m}$, respectively.

previous charging history and the velocity of a grain through the plasma; the equations describing charging must be solved simultaneously with the equation of motion. We found the characteristic surface potential, independent of the particle size, to be $\Phi \approx -30 \text{ V}$ in the cold plasma torus ($4R_J < r < 6R_J$) and $+3 \text{ V}$ elsewhere (M.H., G.M. and E.G., manuscript in preparation). (A surface potential of $+1 \text{ V}$ on a grain $1 \mu\text{m}$ in radius is a result of 700 missing electrons; for isolated grains the potential and the charge are related as: $\Phi = Q/a$, where a is the radius.)

A charged dust grain orbiting a planet has an equation of motion (in gaussian units in an inertial coordinate system fixed to the planet's centre) of

$$\ddot{\mathbf{r}} = -\frac{\mu\mathbf{r}}{r^3} + \frac{Q}{m} \left(\frac{\dot{\mathbf{r}}}{c} \times \mathbf{B} + \mathbf{E} \right)$$

where $\mathbf{r}$ is the grain's position vector, $\mu = GM_J$, the product of the gravitational constant ($G = 6.67 \text{ cm g}^{-1} \text{ s}^{-2}$) and Jupiter's mass ($M_J = 1.9 \times 10^{30} \text{ g}$), and an over-dot signifies differentiation with respect to time. The first term on the right is the gravitational acceleration. The second term is the Lorentz acceleration where c is the speed of light and $\mathbf{B}$ is the local magnetic field. In an ideal magnetosphere—as described in a rotating frame attached to the planet—there are no electric fields and the magnetic fields remain stationary. The coordinate system transformation from a rotating to an inertial frame results in the 'co-rotational electric field', $\mathbf{E} = (\mathbf{r} \times \boldsymbol{\Omega}) \times \mathbf{B}/c$, with Jupiter's rotation rate $\Omega = 1.76 \times 10^{-4} \text{ s}^{-1}$. Radiation pressure, plasma and neutral drags are neglected as they cause orbital changes only over very long characteristic timescales. The jovian magnetic field has a complicated morphology; in our computer simulations we used the O4+ current sheet model⁵ up to $r=50R_J$ and assumed solar wind conditions outside. To imitate the sector structure of the solar wind, we flipped the sign of the azimuthal component of the interplanetary magnetic field every 14 days. We ignored the jovian tail: particles escaping towards the tail region have little chance of meeting Ulysses.

Initially, let us first ignore the complicated morphology and assume that the magnetic field can be given as that of a simple dipole located at the centre with its magnetic moment aligned with Jupiter's rotational axis. In the equatorial plane $B = B_0(R_J/r)^3$, ($B_0 = 4.2 \text{ Gauss}$) and the field lines pierce the equatorial plane at right angles pointing downwards. The resulting corotating electric field points radially outward, with an amplitude of $5.8 \times 10^{-5} (R_J/r)^2 \text{ V m}^{-1}$. Outside the cold plasma torus, where grains become charged to $+3 \text{ V}$ positive potential, the ratio of the outward pointing force due to the corotating electric field to gravity becomes $1.65 \times 10^{-2}/a_\mu^2$ (a_μ is the grain radius measured in micrometres). Grains escaping Io ($a_\mu < 0.1$) are dominated by electrodynamic forces.

In the case of an aligned dipole, particles started in the equatorial plane remain confined there. The radius of their trajectory circling a magnetic field line is the Larmor radius, $r_L = |\dot{\mathbf{r}} mc / QB|$. For particles ejected from Io (at $r = 5.9R_J$) into the cold plasma torus $r_L = 500R_J a_\mu^2$. The motion of these particles can be described by the guiding centre approximation³ if $r_L |\nabla B/B| < 0.1$. This condition is met for particles with $a_\mu < 0.02$, and grains in this size range remain tied to a magnetic field line and tend to co-rotate with it. Grains in the size range of $0.02 < a_\mu < 0.1$ will enter the hot plasma region where r_L becomes much larger and will be 'centrifuged' out from the magnetosphere. This simple physical picture holds remarkably well in a detailed computer simulation, where we have followed the trajectories and charges of grains in a realistic model of the particles and fields in the jovian magnetosphere (Fig. 1).

As positively charged particles move outward they gain energy from the corotating electric field

$$\epsilon = \int_{6R_J}^{50R_J} EQ dr \approx 0.2 a_\mu \text{ erg}$$

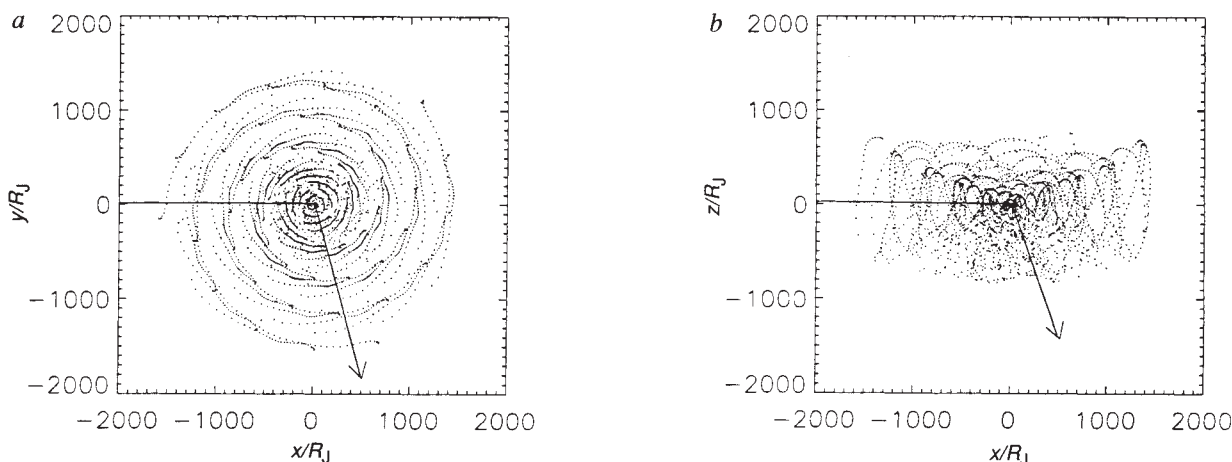


FIG. 3 Scatter plots of 0.1- μm -size grains projected onto the ecliptic plane (a) and to a plane perpendicular to the ecliptic (b). The continuous lines represent the spacecraft's trajectory (our x-axis is rotated in the ecliptic plane to point towards the inbound leg of the trajectory). We have started one particle every 15 minutes and followed the evolution of the entire

growing population of dust grains for 35 days. Each dot represents a dust grain in this snapshot. To a good approximation, this structure expands radially at a constant rate. The number of spiral arms is related to the discontinuities in the ejection longitude and latitude (see Fig. 2).

where the upper limit of the integration is the extent of the magnetosphere in our model. Ignoring the initial gravitational binding energy, the work done by the electric field is the kinetic energy of the ejected dust particle; their exit velocity at the outer boundary of the magnetosphere can be estimated as $v_{\text{exit}} \approx 3/a_\mu \text{ km s}^{-1}$. We therefore expect particles in the size range of $0.03 < a_\mu < 0.1$ to leave the Jovian magnetosphere with a velocity of $100 > v_{\text{exit}} > 30 \text{ km s}^{-1}$. This simple size-velocity relationship is further complicated by the 'tilted' nature of the magnetic field, but it holds within $\pm 10\%$ in our computer simulations (M.H., G.M. and E.G., manuscript in preparation).

The exit direction of an ejected dust particle (with a given mass) is sensitive to Io's position and to the magnetic field geometry (that is, Io's position in magnetic coordinates). To a first approximation, Io's longitude in the inertial reference frame sets the exit longitude and its magnetic longitude determines the exit latitude (Fig. 2). Io's orbital period is 1.77 days, and the period of the magnetic field—Jupiter's rotational period—is 0.414 days. The same magnetic field line sweeps by Io every 0.54 days which is Io's period in magnetic coordinates. A given combination of Io's geographic and magnetic coordinates will be repeated when $1.77n = 0.54m$, where n and m are integers. The smallest number combination to satisfy this condition is $n = 18$ and $m = 59$. Dust grains will leave in the same direction every $P = 32$ days. There are other combinations that give approximate solutions, for example $n = 7$ and $m = 23$ or $n = 11$ and $m = 36$ that result in $P = 12.4$ and 19.5 days, respectively. Portions of the dust beam propagating in the same general direction will be spaced at a distance of $d = v_{\text{exit}}P$. Ulysses encounters the beam with the period of $P'' = v_{\text{exit}}P/(v_{\text{exit}} \pm v_U)$, where $v_U (= 14 \text{ km s}^{-1})$ is Ulysses' velocity in the jovian frame and the $+$ ($-$) sign corresponds to the period before (after) encounter. The smallest grains in our model ($a_\mu = 0.03$) leave the magnetosphere every 32 days with $v_{\text{exit}} = 100 \text{ km s}^{-1}$, so they could produce the observed period of 28 days before the encounter. Similarly, particles with sizes around $a_\mu = 0.06$, leaving the magnetosphere into the same general direction every 19.5 days with velocities around 50 km s^{-1} , could be responsible for the 28-day periodicity after the encounter. These periods are only intended as examples, and further detailed examination of the

data will help to decide the relevant n, m combinations.

In a narrow size range this mechanism will produce a beam of dust particles that swirls around Jupiter like water from a lawn sprinkler (Fig. 3). If we consider a whole size distribution of the grains, the emission morphology becomes complex, resembling the fluffy skirt of a pirouetting ballerina. But the jovian magnetosphere operates as a gigantic mass spectrometer, and most particles will never leave with the proper combination of longitude and latitude to hit Ulysses. The observed quasi-periodicity is still due to particles in a narrow size range.

This simple picture is further complicated by additional perturbations. The magnetic field carried by the solar wind will further spread the grains in the direction perpendicular to the ecliptic plane, and the morphology of the magnetic fields will change as the outer regions of the jovian magnetosphere respond to changes in the solar wind ram pressure. In our numerical experiments we found these to be less important than the contribution of the inner regions of the jovian magnetosphere.

The size and velocity range of the escaping particles in this model are compatible with the Ulysses observations, and the internal periodicity in the production of small grains escaping the jovian magnetosphere might be responsible for the recurrent dust events. More work needs to be done on this model to explain all aspects of the Ulysses dust events, but the main features can be understood. Data from the forthcoming Galileo mission to Jupiter may force further theoretical developments. The process described here assigns a new role to Jupiter as an important source of interplanetary dust particles. Perhaps all giant planets whose moons shed small grains as a result of meteoroid bombardment contribute in a specific size range to the population of interplanetary dust grains. $\square$

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A ferromagnetic transition at 1.48 K in an organic nitroxide

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IN one of the pioneering studies of magnetism at the microscopic level, Heisenberg¹ concluded that ferromagnetism could not exist in compounds consisting only of light elements. A few well defined², purely organic materials (consisting only of the elements carbon, hydrogen, oxygen and nitrogen) have subsequently been found^{3–5} to exhibit evidence of ferromagnetic interactions at low temperatures, with a small number of these showing a transition to a true ferromagnetic state. For example, the *p*-nitrophenyl nitronyl nitroxide radical has a Curie (transition) temperature of 0.60 K (ref. 6), and a possible ferromagnetic transition has been identified^{7,8} in a C₆₀-based ionic complex. More recently, it was reported⁹ that the magnetization and magnetic susceptibility behaviour of a crystalline nitroxide biradical, *N,N'*-dioxy-1,3,5,7-tetramethyl-2,6-diazaadamantane, are indicative of ferromagnetic interactions. Here we report the existence of a ferromagnetic transition in this system, with a Curie temperature of 1.48 ± 0.02 K. As yet, this is the highest transition temperature found for a purely organic, non-ionic material.

This biradical (Fig. 1a) was prepared because an intramolecular ferromagnetic interaction between the two orthogonal N–O groups of the molecule could be expected on symmetry considerations (an unpaired electron is nearly localized on each NO radical)^{9,10}. In the crystal (Table 1; Y. Dromzee, Y. Jeannin, R.C. and A.R., manuscript in preparation) each NO group has three close NO neighbours, one on the other side of the same molecule (i, Fig. 1b) and two (ii, iii, Fig. 1b) on other molecules, suggesting a three-dimensional network of chains interconnected through inter- and intramolecular interactions. The static susceptibility (above 2 K) is characteristic of ferromagnetic interactions⁹ with a positive Curie–Weiss temperature ($\theta = 10$ K) extrapolated from a temperature $T > 150$ K. In the same polycrystal, χT (where χ is magnetic susceptibility) increases when T decreases from 10 K down to 2 K, and the magnetization, M , tends to saturate (87% of the expected saturation value) at 4.2 K

TABLE 1 Structural parameters of *N,N'*-dioxy-1,3,5,7-tetramethyl-2,6-diazaadamantane

(a) Positional and thermal parameters

Atom	<i>x/a</i>	<i>y/b</i>	<i>z/c</i>	<i>U</i> _{eq} [*] (Å ²)
N	0.6318 (3)	0.3764 (2)	0.4037 (2)	0.0411
O	0.6840 (3)	0.4042 (2)	0.5241 (2)	0.0590
C(1)	0.4854 (3)	0.3143 (2)	0.3681 (3)	0.0416
C(2)	0.5000	0.2550 (3)	0.2500	0.0457
C(3)	0.6672 (3)	0.3751 (2)	0.1775 (3)	0.0425
C(4)	0.6551 (3)	0.4373 (2)	0.2941 (3)	0.0399
C(5)	0.5000	0.4970 (3)	0.2500	0.0430
C(6)	0.4797 (4)	0.2552 (2)	0.4874 (3)	0.0583
C(7)	0.8110 (4)	0.4943 (2)	0.3432 (3)	0.0552

(b) Interatomic distance (Å)

Intramolecular			
N–N(i)	3.352 (4)		
N–O(ii)	4.526 (3)		
O–O(i)	5.685 (4)		
Intermolecular			
N–N(ii)	4.895 (5)	N–N(iii)	4.382 (5)
N–O(ii)	4.322 (3)	N–O(iii)	4.341 (3)
O–O(ii)	4.077 (4)	O–O(iii)	4.659 (5)
N–N(iv)	6.283 (3)	N–N(v)	6.283 (3)
N–O(iv)	5.153 (3)	O–O(v)	5.863 (2)
O–O(iv)	5.863 (2)		

Crystallographic system: monoclinic, space group C2/c. Lattice parameters (Å): $a = 8.371$, $b = 14.482$, $c = 10.329$, $\beta = 105.35$. R values for fit: $R = 0.037$, $R_w = 0.035$.

* $U_{eq} = (U_1 U_2 U_3)^{1/3}$ is the equivalent isotropic thermal parameter. We used the anisotropic thermal parameters to refine the atom positions.

(i), (ii), (iii), (iv) and (v) refer to the following equivalent positions relative to the x, y, z set: (i) = $1 - x, y, 1/2 - z$; (ii) = $1 - x, 1 - y, 1 - z$; (iii) = $3/2 - x, 1/2 - y, 1 - z$; (iv) = $x, 1 - y, -1/2 + z$; (v) = $x, 1 - y, 1/2 + z$

and in a field of 5 T.

For the present measurements, single crystals (in the shape of long transparent parallelepipeds with a yellow-orange colour) were prepared as described in ref. 9. No impurities could be detected, except for traces of monoradicals appearing in the frozen electron spin resonance (ESR) spectrum and estimated by spectral reconstitution to be 0.6%. We studied the magnetic properties in low and high magnetic fields, and temperatures between 50 mK and room temperature. The ferromagnetic transition is characterized by a divergence of the a.c. susceptibility, and the onset of spontaneous magnetization at 1.48 K. At

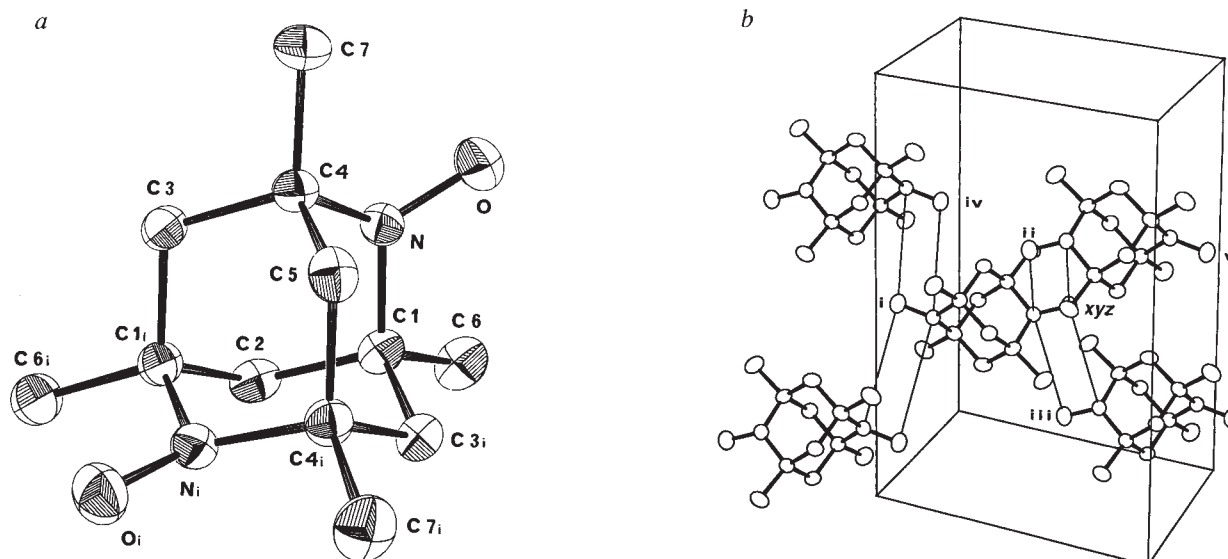


FIG. 1 a, Structure of the *N,N'*-dioxy-1,3,5,7-tetramethyl-2,6-diazaadamantane molecule showing the two NO radicals in opposite positions. b, The

crystals consist of interconnected chains which, given the similar distances between NO near neighbours, may make up a three-dimensional network.

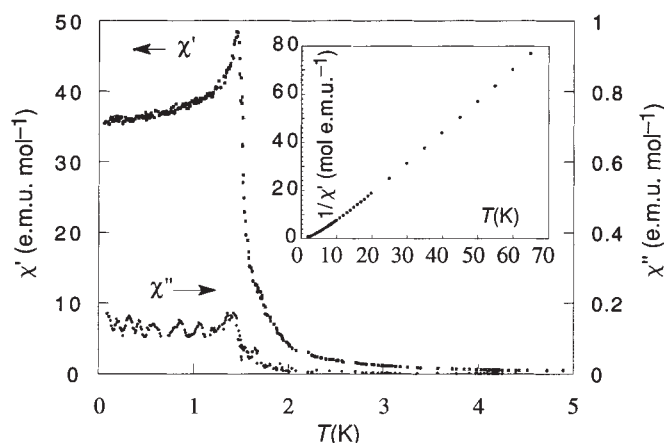


FIG. 2 Alternating current susceptibility (χ' is the real part and χ'' is the imaginary part), showing the divergence of χ' when T approaches T_c from above and its saturation close to $1/N$, the inverse of the demagnetizing field factor. Note the different scale for χ'' , which indicates the existence of very weak magnetic losses below T_c ($\chi''/\chi' \approx 0.01$).

very low temperatures the saturation magnetization gives the expected value for two electrons with spin $\frac{1}{2}$ per molecule. Surprisingly, no hysteresis could be detected.

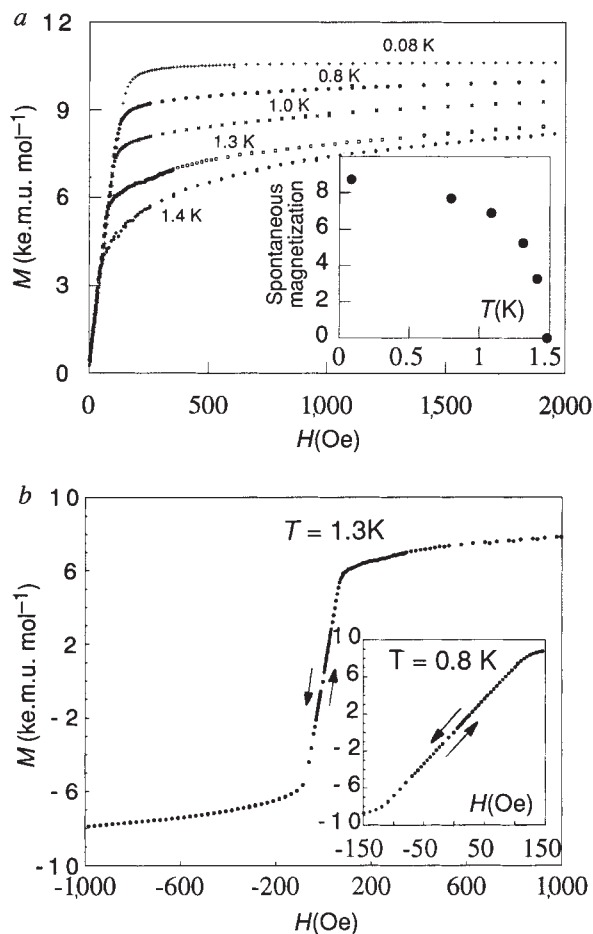


FIG. 3 *a*, Low-field magnetization. This is linear in a low applied field (exactly compensated by the demagnetizing field) and shows the onset of spontaneous magnetization below 1.48 K. The inset shows the temperature dependence of the spontaneous magnetization taken at the departure from linearity of the $M(H)$ isotherms. *b*, The low-field hysteresis cycle at 1.3 K (0.8 K for the inset) has no detectable hysteresis (within 0.1 Oe).

The low-temperature susceptibility, χ_{ac} , of single crystals (several pieces measuring about $1 \times 0.5 \times 0.5$ mm) was measured in a mutual inductance coil where an alternating current creates a small a.c. field (638 Hz and ~ 0.05 Oe peak to peak) in the sample. The real and imaginary parts of the a.c. susceptibility are shown in Fig. 2. The real part, χ' , deviates from the high-temperature trend below 70 K, and a plot of $1/\chi'$ against T is no longer linear (see inset to Fig. 2) although a linear extrapolation would still give a positive value of θ . Below 4 K, χ' increases rapidly until it reaches a limiting value at 1.48 K. This limit corresponds to the inverse of the demagnetizing field factor for a ferromagnet. The ferromagnetic behaviour was confirmed by low-field magnetization measurements on single crystals (several crystals with long dimension parallel to the field were cooled in a miniature dilution refrigerator). Below T_c and in low field, the magnetization is linear with field (Fig. 3) with a slope equal to the inverse of the demagnetizing field factor. This is typical of ferromagnetic systems, where the magnetization M is equal to H/N (N is the demagnetizing field coefficient) as long as the applied field is exactly compensated by the demagnetizing field.

The results indicate the existence of a spontaneous magnetization below a critical temperature T_c , which sets in at the departure from the linear regime of $M(H)$ and has the thermal dependence shown in the inset to Fig. 3*a*. Moreover, the temperature dependence of the low-field d.c. susceptibility, M/H , taking into account the different demagnetizing coefficients, is only slightly larger than that of the a.c. susceptibility. This is different from what is usually observed in hysteretic systems, where χ_{ac} drops below T_c because of time-dependent effects. The losses associated with the magnetic viscosity, as seen in the imaginary part, χ'' , of the a.c. susceptibility (Fig. 2), are very small, in agreement with the small difference between M/H and χ_{ac} (which correspond to measuring times of ~ 50 s and 0.002 s, respectively). This is in agreement with hysteresis curves measured in fields $H < 150$ Oe: no hysteresis could be detected (Fig. 4), and the coercive field must be smaller than 0.1 Oe. A more careful study of the ferromagnetic transition itself (determination of critical exponents close to T_c) should be made on larger ellipsoidal crystals.

The low-field measurements are thus characteristic of ferromagnetic behaviour (divergence of the a.c. susceptibility and onset of spontaneous magnetization at 1.48 K) but have two particular features: the absence (or very small value) of hysteresis and the similar values for d.c. and a.c. susceptibilities. Magnetization measurements with the field perpendicular to the

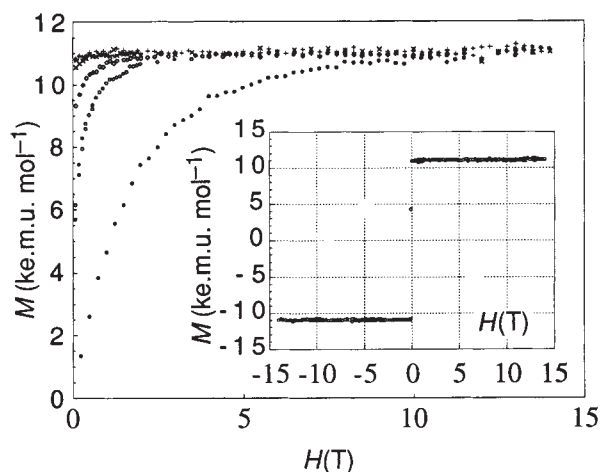


FIG. 4 High-field magnetization at temperatures of 4.20, 1.64, 1.04, 0.50 and 0.10 K gives the expected total saturation which could not be reached in previous experiments at 4.2 K and in a field of 5 T (ref. 9). The inset (for $T = 0.1$ K) confirms that no hysteresis is observed even after applying a 14-T field.

long dimension of the crystals mainly differ from the previous ones by the effect of different demagnetizing field factors. The long-range ferromagnetic order is thus three-dimensional.

High-field magnetization measurements have been made (in collaboration with J. Voiron, L. Neel Laboratory, Grenoble) at fields up to 14 T in a superconducting coil using the same miniaturized dilution refrigerator. They confirm the low-field spontaneous magnetization and reversibility (Fig 4.). The 0.1 K curve clearly shows 100% saturation for spin $\frac{1}{2}$ of the NO radicals. The results agree with previous experiments showing 87% saturation at 4.2 K and in 5 T (ref. 9).

We conclude that it is possible to have intramolecular ferromagnetic interactions between nitroxide radicals in crystals of *N,N'*-dioxy-1,3,5,7-tetramethyl-2,6-diazaadamantane. The intermolecular interaction is also ferromagnetic and gives rise to truly three-dimensional ferromagnetic order at a Curie temperature of 1.48 K. The very weak magnetic losses detected in

a.c. susceptibility measurements are consistent with the extremely narrow hysteresis cycle (smaller than 0.1 Oe). □

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Foraminiferal boron isotope ratios as a proxy for surface ocean pH over the past 21 Myr

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THE pH of the surface ocean is a sensitive function of its alkalinity and total inorganic carbon concentration, properties which also control the partial pressure of atmospheric carbon dioxide¹⁷. Thus, an accurate proxy for past ocean pH could yield information about variations in atmospheric CO₂. Recently, it has been suggested that the boron isotopic composition of foraminiferal tests depends on the pH of sea water as well as its isotopic composition^{1,2}. Here we present boron isotope and elemental data for sedimentary pore fluids and isotope data for bulk foraminiferal samples from a deep-sea sediment core. The composition of the pore waters implies that sea water boron concentrations and isotopic composition have been constant during the past 21 Myr, allowing us to reconstruct past ocean pH directly from the foraminiferal isotope data. We find that 21 Myr ago, surface ocean pH was only 7.4 ± 0.2 , but it then increased to 8.2 ± 0.2 (roughly the present value) about 7.5 Myr ago. This is consistent with suggestions^{3–5} that atmospheric CO₂ concentrations may have been much higher 21 Myr ago than today.

Pore fluid and foraminifera samples were isolated from Ocean Drilling Program (ODP) hole 803D (2° 25.98' N, 160° 32.46' E; water depth, 3,410 m). This core is composed of >99% biogenic sediment, carbonate ooze from the top of the core to ~220 m below the sea floor where it becomes carbonate chalk, except for the basal few metres⁶. Bulk foraminifera greater than 150 µm in size were hand-picked and two splits were analysed in parallel. A fraction of each sample was examined by scanning electron microscopy to check for non-foraminiferal sedimentary components and secondary carbonate crystals. Our criterion for analytical reliability, for foraminifera, was that the measured B/Ca ratio for each sample pair agreed within 0.5%. Only pairs that met this criterion were analysed for isotopic composition. Although the microscopical examinations indicate that the observed foraminiferal $\delta^{11}\text{B}$ trend is not the result of contamination by morphologically identifiable phases, it remains a possibility that it is the result of post-depositional isomorphous diagenesis and recrystallization. This question can be resolved through the analysis of additional cores.

There are no significant trends in either B concentrations or

TABLE 1 Boron concentration and $\delta^{11}\text{B}$ data for ODP hole 803D

Depth (mbsf)	Pore fluid		Foraminifera $\delta^{11}\text{B}$
	$\mu\text{mol l}^{-1}$	$\delta^{11}\text{B}$	
8.5	459	34.6	16.2
18	467		
27.5	551		
37.0	484		
46.5	442		
52.1	474		
65.1			17.0
80.6	471	33.0	
109	477		
137	493		
141			
166	467		14.8
194	450	35.5	
198			11.5
221	439		
250	472		
277	445		
294			10.5
309	466		
339	467	34.0	
364	445		
394	469		
452	411		
478	451	34.5	
508	450		
545	450		
623	451	33.0	

Boron isotope compositions are reported as per mil (‰) deviations in the $^{11}\text{B}/^{10}\text{B}$ ratio from NBS 951. Pore fluids were sampled using standard squeezing methods and concentrations were determined by colorimetry^{22,23}. All isotope ratios were determined by thermal ionization mass spectrometry as the negative molecular ion, BO_2^- , following the whole sample integration method of You *et al.*¹³. For foraminifera, the uncertainty of isotopic compositions is ~1‰, based on the analysis of parallel samples, and 0.6‰ for pore fluids based on duplicate seawater analyses. Actual scatter in the pore fluid data is slightly larger, most probably because of adsorption/desorption effects associated with sampling. mbsf, metres below sea floor.

$\delta^{11}\text{B}$ values of the pore fluids throughout the core (Fig. 1a, b). Average concentrations are 9.0‰ higher and average $\delta^{11}\text{B}$ values are 5‰ lower than modern bottom water at this site (420 $\mu\text{mol l}^{-1}$ and 39.5‰). These offsets are most probably an artefact of pore fluid extraction at temperatures and pressures other than those *in situ*. It is known that B adsorption coefficients are pressure- and temperature-dependent, and that the B adsorbed on marine sediment is ~25% depleted in $\delta^{11}\text{B}$ (ref. 7).

Although sedimentary pore fluids are samples of buried sea

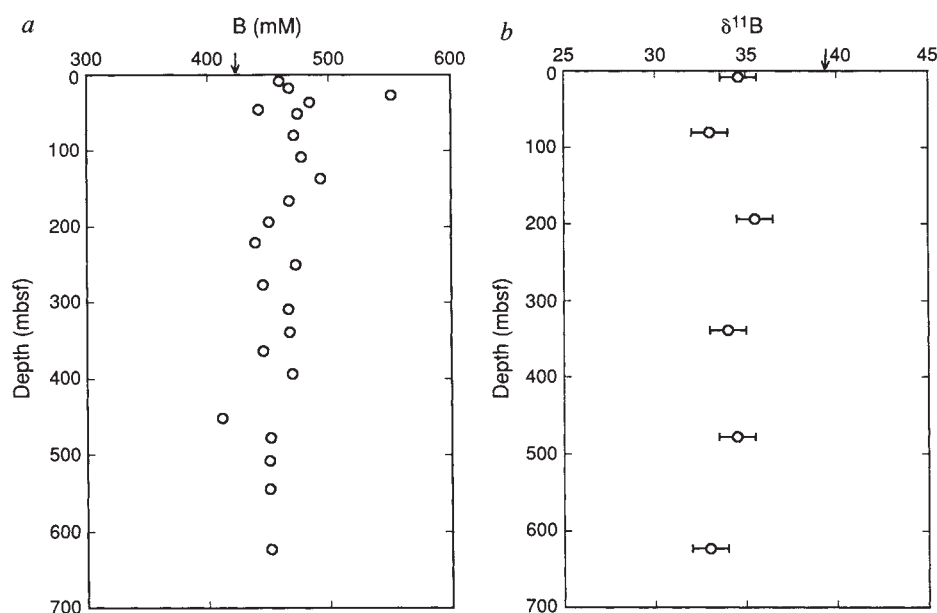


FIG. 1 Pore fluid data for ODP hole 803D. *a*, Boron concentrations against depth. *b*, $\delta^{11}\text{B}$ against depth. Arrows indicate bottom seawater values. mbsf, metres below sea floor.

water, their compositions may be altered as a result of reactions and transport⁸. In general, they do not record the composition of sea water that is coeval with the surrounding sediment layer. The essentially constant concentrations and isotopic compositions, in this core, argue against significant reactions affecting B distributions. We therefore need consider only transport, both convective and diffusive, in using this data for reconstructing seawater B compositions. Convective transport is known to be common until sediments reach a thickness of ~300 m (21 Myr ago at this site) after which convection ceases and transport becomes controlled by diffusion⁹. Elemental distributions of Mg, Ca and Cl in this core do in fact indicate that transport has been diffusion-dominated for at least 21 Myr (refs 10, 11). A finite-difference diffusion model, based on the assumption that transport was convection-dominated before 21 Myr ago and has been diffusion-limited since, constrain seawater elemental and $\delta^{11}\text{B}$ values at 21 Myr to be within 4% and 1.5% of modern values. The model uses published physical properties and adsorption coefficients determined in our laboratory for sediments from hole 803D⁶. Large adsorption coefficients effectively reduce diffusion scale lengths, allowing seawater B compositions to be preserved. The relative invariance over this period, which is roughly equal to the seawater residence time of B, implies that the seawater B isotopic composition and concentration may be effectively buffered, most probably by reaction with the oceanic crust (refs 12–14, and H.J.S., A.J.S. and H. Standigel, manuscript in preparation). The constancy of B concentrations reinforces the likelihood of constant isotopic compositions as changes in isotopic composition would be expected to be associated with changes in concentration.

This constancy of seawater $\delta^{11}\text{B}$ over the past 21 million years allows variations in foraminifera compositions to be interpreted as changes in the isotope fractionation between sea water and foraminifera. Fractionation factors derived from this data set are constant, $-23.5 \pm 1.5\%$, over the past ~7.5 Myr. But between 7.5 and 21 Myr the magnitude of the fractionation increases to $-29.5 \pm 1.5\%$ (Fig. 2). Hemming *et al.* observed that the $\delta^{11}\text{B}$ of a variety of modern carbonates (biogenic and abiogenic, not including foraminifera) were nearly uniformly $-21 \pm 1\%$ fractionated from sea water and argued that this lack of species-dependent fractionation is consistent with inorganic equilibrium. Vengosh¹ reported values of $-23.5 \pm 3\%$ for five separate modern planktonic foraminifera species⁴. Our data for modern foraminifera indicate a similar fractionation from sea water, $-23.5 \pm 1\%$, implying that the seawater–foraminifera fraction-

ation is also principally controlled by inorganic equilibrium.

Equilibrium isotopic fractionation of B between two phases is thought to result mainly from differences in the number and energies of vibrational modes associated with the trigonal and tetrahedral oxyboron species and the relative fraction of these

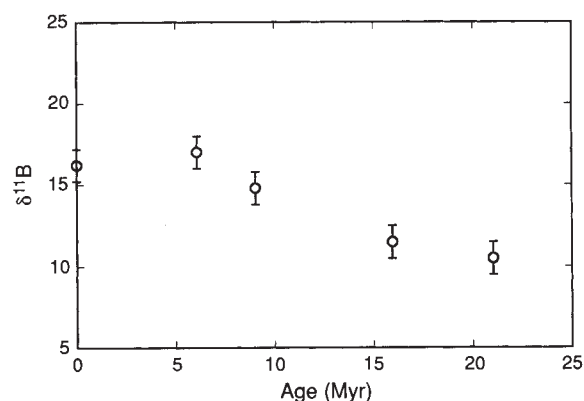


FIG. 2 Boron isotopic composition of foraminifera as a function of age.

species in each phase^{12,15}. In solution, speciation is a function of pH and it has been argued that in carbonates a four-coordinate species dominates; the seawater–foraminifera fractionation is thus expected to be pH-dependent^{4,5}. Palmer *et al.*¹⁶ experimentally determined fractionation factors associated with adsorption of B on clay from sea water as a function of pH and interpreted the results in terms of changes in coordination. Using their experimental relationship as an analogue for seawater–foraminifera fractionation, at a typical pH of 8.25 for the surface ocean, the expected fractionation is $-23.7 \pm 0.7\%$, indistinguishable from the observed value. Accepting that pH exerts a first-order control on the seawater–foraminifera fractionation and using the relationship established by Palmer *et al.*, we can calculate surface-ocean pH values over roughly the past 21 Myr.

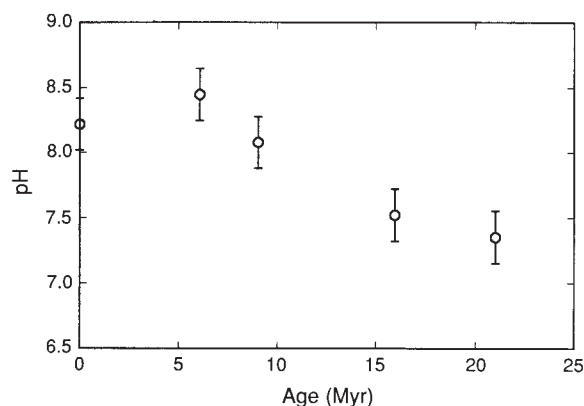


FIG. 3 Calculated pH values of surface sea water as a function of age. Values are based on the assumption that the B isotopic composition of sea water has been constant since 21 Myr ago and on the relationship between pH and isotopic fractionation established by Palmer *et al.*¹⁶. A best-fit line to their experimental isotope fractionation data between pH 6.70 and 8.45 is: fractionation (‰) = $-72.6 + 5.93 \times \text{pH}$ with $R = 0.96$ and number of samples $n = 15$.

Calculated values are 8.2 ± 0.2 between the present and 7.5 Myr, decreasing to 7.5 ± 0.2 at 12.5 Myr and 7.4 ± 0.2 at 21 Myr ago (Fig. 3).

These pH variations imply changes in surface-water alkalinity and/or total inorganic carbon concentrations. In turn, surface-water variations in these properties can result from changes in whole-ocean average values and/or changes in their vertical distribution. Whole-ocean alkalinity and total inorganic carbon are functions of both continental and oceanic crust weathering and carbonate metamorphism rates, and their distribution within the ocean is driven by the imprint of biological processes on circulation¹⁷. Net productivity in surface waters and respiration in deeper waters results in a range of pH values from ~ 8.2 in typical surface waters to as low as 7.5 in parts of the deep ocean. Hence, the estimated seawater pH record implies considerable changes in global weathering and metamorphism and/or oceanic biogeochemical cycles, processes that also determine the atmospheric partial pressure of carbon dioxide^{17,18}.

Methods for reconstructing the CO_2 content of the ancient atmosphere, based on the carbon isotopic composition of marine organic matter and soil carbonates, have recently been developed. Using the isotopic composition of organic matter, Arthur *et al.*^{3,4} have argued that the atmospheric partial pressure of CO_2 was 4.5 times the present value at 21 Myr ago, implying (as do our pH estimates⁵) that there have been changes in the surface-water carbon system. Soil isotopic data are consistent with higher CO_2 partial pressures 21 Myr ago, although within estimated uncertainties they do not rule out smaller variations⁴.

If both CO_2 partial pressures and pH are known, all chemical species in the aqueous carbon system including alkalinity and total inorganic carbon can be calculated. For example, accepting the value of 4.5 times atmospheric CO_2 with a pH of 7.4 ± 0.2 , then 21 Myr ago total carbon and alkalinity would have been $\sim 0.6 \pm 0.2$ times present-day values. Following the development of more detailed records, these properties could be used to constrain carbon-system geochemical models which have recently been the subject of intense debate^{18–21}. □

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Ultra-depleted primary melt included in an olivine from the Mid-Atlantic Ridge

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MODELS of magma genesis at mid-ocean ridges¹, together with recent experimental data² and observations of trace element abundances in clinopyroxenes from abyssal peridotites³, suggest that small-volume melt fractions can be efficiently extracted from the melting mantle. As shown in ref. 3, residues of this type of melting (fractional melting) display extremely low abundances of incompatible trace elements and extreme fractionation amongst them, especially at advanced stages of the process because of the compounded effects of differences in the partition coefficients. If this process operates beneath mid-ocean ridges, one would expect to sample melts that are correspondingly depleted and fractionated in trace elements. Indeed, the existence of very depleted melts in mid-ocean ridges was predicted previously^{4–6} in order to explain the presence of magnesian pyroxene and calcic plagioclase in mid-ocean-ridge basalts. We report here the discovery of melt that has major and trace element characteristics consistent with these predictions^{4–6}, occurring as an inclusion in an olivine phenocryst in a typical mid-ocean-ridge basalt from the Mid-Atlantic Ridge. Although our preferred model for the origin of this 'ultra-depleted' melt is critical (continuous) melting, we cannot at this stage rule out other models. Our results underscore the importance of trapped melt inclusions as recorders of the processes involved in melting and melt extraction, and also as pointers to primary melt compositions.

The basalt sample in which the ultra-depleted melt (UDM) inclusion was found was dredged from the axial valley in an undisturbed segment of the Mid-Atlantic Ridge between the Doldrums and Vema fracture zones (9° N, 39.5° W). Details of dredging and of mineralogy and melt inclusions in minerals have been presented elsewhere⁷. In brief, the sample consists of ~ 20 –30% phenocrysts of olivine (Fo_{90–87}), plagioclase (An_{86–80}), clinopyroxene (Mg# 89–87) and spinel (Cr# 38–47) in a matrix glass of slightly evolved composition (Table 1).

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TABLE 1 Major element compositions of phases

	Glass*	Quench cpx	Fe-rich opx	Liquid†	UDM (initial composition)	Host olivine	Opx inclusion	Spinel inclusion	Matrix glass	Glass inclusions (initial composition)		Expt liquid‡
SiO ₂	54.5	51.8	55.8	54.4	52.9	40.6	55.3	0.04	49.7	51.7	50.2	53.4
TiO ₂	0.34	0.21	0.04	0.32	0.29	0.00	0.04	0.16	1.25	0.48	0.56	0.36
Al ₂ O ₃	18.5	5.1	2.02	17.3	15.5	0.04	2.1	39.0	15.9	17.5	17.9	15.82
Fe ₂ O ₃	nd	nd	nd	nd	0.7	nd	nd	5.6	nd	0.7	0.7	nd
FeO	6.5	5.2	6.58	6.45	6.4	9.8	6.1	8.8	9.2	5.9	6.3	6.74
MnO	0.09	0.08	0.13	0.09	0.08	0.15	0.13	0.13	0.20	0.09	0.11	nd
MgO	4.7	17.8	32.4	6.29	10.5	48.7	33.6	19.1	8.1	9.3	9.8	9.75
CaO	14.3	18.3	2.12	14.0	12.5	0.29	2.2	nd	11.8	12.2	12.3	12.77
Na ₂ O	1.35	0.15	0.06	1.25	1.12	0.00	0.06	nd	2.58	2.11	1.98	1.03
K ₂ O	0.00	0.00	0.00	0.00	0.00	0.00	0.00	nd	0.10	0.01	0.01	nd
Cr ₂ O ₃	0.03	0.70	0.41	0.07	0.07	0.09	0.41	26.8	nd	nd	nd	0.21
Mg#	56.3	86.0	89.9	63.5	74.5	89.9	90.8	79.5	61.1	73.8	73.5	74.12

* Glass (UDM before corrections).

† Liquid after the first step of correction.

‡ Experimental liquid at 5 kbar and 1,250 °C (T-827) from ref. 19.

Mg# = $100 \times \text{Mg}/(\text{Mg} + \text{Fe}^{2+})$ in mole. Electron probe and analyses were made at the Vernadsky Institute, Moscow. Ferric iron in spinel was calculated on the basis of stoichiometry. Ferric iron in melts were calculated assuming wüstite-magnetite buffer (ref. 11). nd denotes not determined.

The UDM inclusion studied here was found in a 1-mm fragment of olivine phenocryst and has been described elsewhere⁸. The host olivine is uniform with $\sim\text{Fo}_{90}$, and contains at least 10 melt inclusions of dimension $\sim 100\text{--}10\text{ }\mu\text{m}$ and a few brown spinel inclusions. The glass inclusions generally have sub-spherical/ellipsoidal shapes with boundaries modified to a sub-planar 'negative crystal'-like outline, which can be parallel to the extinction direction of the host. The distribution of the inclusions within the host crystal does not look like it is controlled by sealed cracks, which might be expected for inclusions of secondary origin⁹. Figure 1 shows that the UDM inclusion is $\sim 60 \times 40\text{ }\mu\text{m}$ in size and consists of clear glass ($\sim 80\%$ in volume), a large euhedral crystal of orthopyroxene (opx) with very well developed facets, and feathery clinopyroxene (cpx) quench crystals grown on the opx. Table 1 summarizes chemical compositions of the phases. Other melt inclusions in the same olivine grain consist only of glass and shrinkage bubbles. Judging from the size of the opx crystal in the inclusion and the absence of opx in coexisting inclusions, we speculate that the opx was trapped in the host olivine together with the melt, rather than growing from the melt after entrapment. We consider that this inclusion is a primary melt inclusion rather than a melted polyphase crystalline inclusion. For instance, the opx within the inclusion is euhedral and unzoned (except for the quench rims) and does not show evidence for an unmelted precursor crystal. The host olivine is also unzoned and does not show evidence for resorption. The composition of the opx (Table 1) is consistent with being in equilibrium with the host olivine. It can be seen in Fig. 1, as has been observed previously⁸ that the olivine wall surrounding the inclusion is richer in Fe than the host, indicating crystallization of olivine during quenching. Similar narrow, Fe-rich rims grown during quenching were observed on the opx. The cpx is evidently the result of quench crystallization within the inclusion.

The initial major element composition of the UDM was calculated in two steps. First, corrections for Fe-opx and quench cpx were made by adding 3.7 wt% of Fe-opx and 4.4 wt% of quench cpx to 91.9 wt% of the measured melt (Table 1). These proportions were derived previously⁸. Second, starting with the corrected melt composition, we corrected for post-entrapment growth of the host olivine by adding equilibrium olivine in 0.1 wt% increments to the melt and recalculating the equilibrium olivine composition, iterating until the equilibrium olivine composition became identical to the host olivine. The calculations used the formulation of ref. 10 with the oxidation state of iron corresponding to the wüstite-magnetite buffer¹¹. As shown in Table 1, a 10 wt% correction was needed for deriving the initial

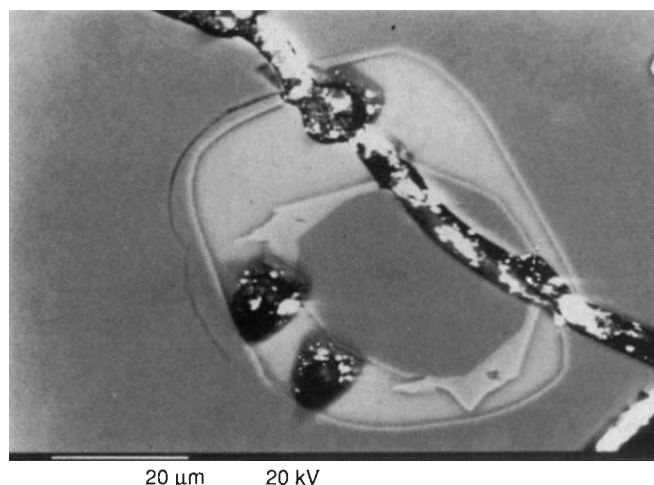


FIG. 1 Back-scattered electron image of the UDM inclusion in olivine. A fracture through the specimen has been enhanced by repeated polishing during the course of the study. Fe-rich olivine crystallized after entrapment can be seen as a thin brighter layer all the way along the wall of the inclusion. A large crystal with angular outline within the inclusion is opx, and Fe-rich rims can also be seen around it. Quench cpx occurs as irregular crystals attached to the sides of the opx. Bright speckles are remains of gold coating for ion probe analysis. Two ion probe pits are visible.

UDM composition. For other inclusions in the same olivine host, only the latter correction was applied. Plagioclase, which could be expected to be in equilibrium with the glass, is not present in the inclusion, probably because of difficulty in nucleation or other kinetic factors.

Trace elements in the UDM inclusion were analysed with a Cameca IMS 3f ion microprobe at Woods Hole Oceanographic Institution, using techniques described elsewhere^{3,12}. In brief, energy-filtered secondary ion intensities of trace elements were converted to concentrations, using empirical relationships between intensity and concentration established from basalt glass standards. The accuracy and precision of the results are believed to be within $\pm 10\%$ for Ti, V, Cr, Sr, Y, Zr, Dy, Er and Yb, around $\pm 20\%$ for Ce, Nd, Sm and Eu, and $\pm 40\%$ for La. Two separate spots analysed in the UDM inclusion were identical within the analytical uncertainties. As the Ti content obtained by ion microprobe was close to that calculated in the initial melt composition, and in view of the analytical uncertainties, we did not make corrections for the post-entrapment crystallization.

The trace element chemistry of the UDM (Table 2) is unique in that all incompatible elements (except Ti and Na) are extremely depleted and that fractionation amongst incompatible elements is also extreme. For instance, Sr (1.3 p.p.m.) and Zr (2.9 p.p.m.) are in strong contrast to the values of 113 p.p.m. and 104 p.p.m., respectively, in average, normal mid-ocean-ridge basalt (NMORB)¹³; even very primitive MORB glasses (N.S. and A.V.S., unpublished results) have concentrations more than a factor of 15 higher than these. $[La/Sm]_N$, a measure of rare-earth element (REE) fractionation, is 0.04 in the UDM, as opposed to 0.65 in average NMORB¹³ and 0.35 in primitive MORB (unpublished results). Furthermore, Ti/Zr is very high (594), compared with 93 in average NMORB¹³ or 160 in primitive MORB (unpublished results), and adds to the uniqueness of the UDM. REE characteristics are illustrated in Fig. 2.

The major element composition of the UDM meets the petrological criteria for equilibrium with mantle minerals. First, the Mg# (75) is high enough for equilibration⁴. The formulations of Kinzler and Grove¹⁴ indicate that the UDM can be in equi-

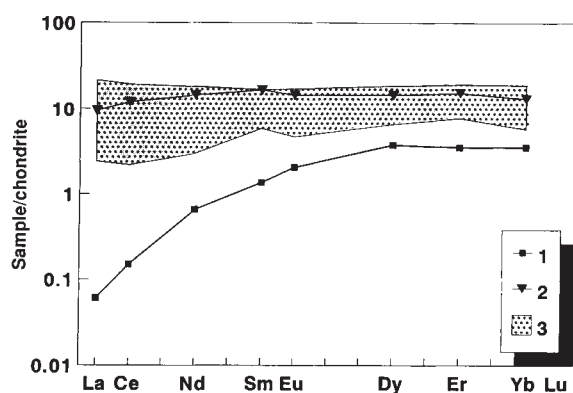


FIG. 2 Chondrite-normalized REE patterns of the UDM (■), the matrix glass (▲), and a range of primitive MORB glasses and glass inclusions (hatched area). Glass data are from unpublished results (A.V. and N.S.), and normalizing factors from ref. 18.

ilibrium with plagioclase lherzolite assemblage at 4.6 ± 1.6 kbar and $1,234^\circ\text{C}$. Furthermore, the UDM composition is similar to a liquid (T-827) experimentally produced at 5 kbar, $1,250^\circ\text{C}$ from the Tinaquillo lherzolite¹⁹ as shown in Table 1. This liquid is in equilibrium with a lherzolitic assemblage. The fact that the UDM composition is slightly more refractory than T-827 indicates that the UDM can be in equilibrium with a harzburgitic assemblage. We therefore suggest that the UDM is a primary mantle melt in equilibrium with a lherzolite/harzburgite residue. The petrological criteria support our contention that the UDM inclusion is a primary melt inclusion, rather than a melted polyphase crystalline inclusion.

Two of the melt inclusions in the same olivine were analysed for major elements. They were subsequently ground away completely in the process of exposing the UDM inclusion and were not available for ion probe analysis. Their initial compositions (Table 1) have characteristics intermediate between the UDM and the matrix glass.

The geochemical characteristics of the UDM could be explained by a number of melting and melt extraction models. It is evident, however, that a simple single-stage batch melting process from a fertile mantle source cannot produce the required depletions in and fractionations amongst incompatible elements (for example, the ratios La/Sm and Ti/Zr).

One of the possible mechanisms is critical (continuous) melting, whereby small-degree melting is followed by melt extraction, with some melt retained in the residue; unlike fractional melting in which melt is completely extracted, the retained melt efficiently buffers highly incompatible elements from being fractionated too much. This type of melting process was first pro-

TABLE 2 Trace element concentrations

	UDM (p.p.m.)	Matrix glass (p.p.m.)
La	0.015	2.24
Ce	0.09	7.24
Sr	1.3	86.5
Nd	0.30	6.50
Zr	2.9	60.6
Sm	0.21	2.44
Eu	0.12	0.81
Ti	1,722	6,155
Dy	0.93	3.53
Er	0.62	2.43
Y	10.2	20.4
Yb	0.57	2.15

posed by Dick¹⁵ and developed later by Langmuir *et al.*¹⁶ as continuous melting and by Maaloe¹⁷ as critical melting. Our model assumes that mantle can retain only a certain amount of melt, and that melting proceeds in a closed system until this level is reached. Above the critical level, the system can lose any small amount of excess melt. It is also assumed that the critical amount of melt is always retained in the residue. The UDM can be satisfactorily modelled as an instantaneous melt fraction at 17 wt% melting of a depleted mantle with 2.2 wt% retained melt. Most of the UDM characteristics can also be produced by mixing melt fractions produced by fractional melting. A multi-stage melting model⁵, in which the increments are fairly small and some melt is retained, can also adequately explain the UDM characteristics. In all possible mechanisms however, extraction of melt must follow each stage (or increment) of melting. Details of these melting models will be published elsewhere.

As mentioned earlier, the UDM could be in equilibrium with a lherzolite or possibly a harzburgite residue at pressures of ~ 5 kbar. This pressure is unusually low—pressures of ~ 10 – 20 kbar are usually discussed²⁰. This suggests that the UDM could be the last fraction of melt produced in an ascending mantle column, representing the most advanced stage of critical (continuous) melting at the shallowest level. The matrix glass, on the other hand, could be derived from integration (polybaric mixing) of pre-existing melt fractions as suggested here. We further speculate that the chemical spectrum in primary MORB magmas is established among individual instantaneous melt fractions during critical (continuous) melting. Subsequent integration (mixing) to various degrees at decreasing depth would then produce a variety of parental MORB magmas. □

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Bioluminescent symbionts of flashlight fishes and deep-sea anglerfishes form unique lineages related to the genus *Vibrio*

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BIOLUMINESCENT symbioses range from facultative associations to highly adapted, apparently obligate ones¹. The family Anomalopidae (flashlight fishes) encompasses five genera of tropical reef fishes that have large suborbital light organs². The suborder Ceratioidei (deep-sea anglerfishes) contains 11 families. In nine of these, females have a bioluminescent lure^{3,4} that contains bacterial symbionts⁵. In all other fish light-organ symbioses (occurring in 10 families in 5 orders⁶), the symbionts belong to three *Photobacterium* species⁷; nonsymbiotic luminous bacteria are *Vibrio* species⁸. The bacteria are extracellular and tightly packed in tubules that communicate with the exterior⁷, releasing bacteria into the gut of the host or the surrounding sea water. The released bacteria are usually cultivable and can contribute to planktonic populations^{9,10}. Although anomalopids release bacteria⁹ and ceratioids have pores that would allow release, the fate of these bacteria is unknown and they cannot be cultured by standard isolation techniques. We report here phylogenetic analysis of 16S ribosomal RNA gene sequences from light organs that show that anomalopid and ceratioid symbionts are not known luminous bacteria, but are new groups related to *Vibrio* spp. They are characterized by host specificity, deep divergence between symbionts from different genera (anomalopids) or families (ceratioids) and,

possibly, parallel divergence of hosts and symbionts.

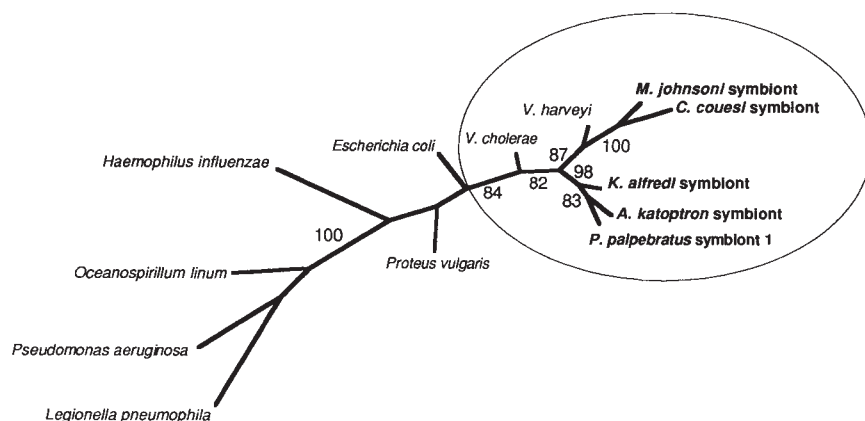
In this study new 16S rRNA gene sequences were obtained (Table 1) and phylogenetic analysis was used to investigate the nature of the anomalopid and ceratioid symbionts. Inability to culture the bacteria could result from different mechanisms in the two groups. The symbionts may undergo irreversible physiological differentiation within the light organ, rendering them incapable of growth under culture conditions that might otherwise support growth of the undifferentiated cells. In this case they may be closely related to *Vibrio* or *Photobacterium* spp. Alternatively these symbionts could have genetically changed, losing adaptations that allow growth in nonsymbiotic environments and laboratory culture conditions, during a long period of obligate symbiosis in isolation from free-living populations. In this case one might expect deep divergence, and parallel divergence between symbionts and hosts. Our results support the latter hypothesis.

Polymerase chain reaction (PCR) amplification of 16S rRNA genes using universal eubacterial primers¹¹ followed by direct sequencing¹² allowed us to obtain data from light organs without cultivation of the bacteria. The anomalopid and ceratioid light organs contain pure cultures of symbionts; only one type of symbiont could be detected in a given light organ as shown by previous work^{13–16}. Direct sequencing of 16S rRNA gene PCR products revealed only a single sequence. But low levels of a cosymbiont (<10% of the major symbiont population) could go undetected by these methods.

Parsimony analysis of the symbionts and representative γ -3 proteobacteria using PAUP shows that the anomalopid and ceratioid symbionts group with species in the genus *Vibrio* (Fig. 1). A similar result was obtained in a maximum likelihood analysis with fastDNAm1 (bootstrapped 100 times with input taxa jumbled, global rearrangement and a transition:transversion ratio of 2), with the exception that *V. cholerae* cannot be placed with confidence. The group containing the anomalopid and ceratioid symbionts and *V. harveyi* is supported at 92% in the maximum likelihood analysis. These results show that the anomalopid and ceratioid symbionts belong to species in the genus *Vibrio*, or new, allied genera analogous to *Photobacterium*. Thus the anomalopid and ceratioid symbionts appear to have arisen from the same common ancestor that was the source of the facultative *Photobacterium* light organ symbionts and other free-living luminous marine *Vibrio* spp., and the physiological differences that make them difficult to culture are probably due to evolution occurring after the establishment of symbiosis, rather than to a dramatically different phylogenetic origin.

The ceratioid and anomalopid symbionts do not belong to

FIG. 1 Parsimony tree of γ -3 proteobacteria produced with PAUP with *Legionella pneumophila* (γ -2 proteobacteria) as the outgroup. The dataset used included 1,201 positions. The heuristic search algorithm was used with a transversion weight of 2. The dataset was bootstrapped 1,000 times with the starting tree constructed by random addition for each replication. Numbers beside branches are the per cent of bootstrap replications in which the group indicated appeared in the shortest tree (only groups supported at >80% are labelled). METHODS. DNA was prepared as in refs 13 or 22. Table 1 contains sources for sequences. Collection data can be found in refs 15 and 16, with the exception that the *K. alfredi* sample was collected at Roatan, Honduras in 1990. Amplification and sequencing were according to refs 12 and 16. Sequence from each primer was read through the next priming site, so sequencing runs overlapped, except in the case of *P. palpebratus* 2, which was partially sequenced. Sequences were aligned to the *Escherichia coli* 16S rRNA secondary structure, checked for complementarity of helical regions and analysed with the Olsen phylogenetic analysis programs^{23–25}, PAUP²⁶,



PHYLP 3.41²⁷ and fastDNAm1²⁸. Indels and hypervariable regions that could not be reliably aligned were excluded from analysis.

TABLE 1 Sequences used for analysis

Bacterial species	Culture collection or host	Accession number	Total positions	Reference or source
<i>Legionella pneumophila</i>	ATCC 33152	M59157	1,518	19
<i>Pseudomonas aeruginosa</i> NIH 18	ATCC 25330	M34133	1,496	19
<i>Oceanospirillum linum</i>	ATCC 11336	M22365	1,526	19
<i>Haemophilus influenzae</i>	ATCC 33391	M35019, M59433	1,487	19
<i>Proteus vulgaris</i>	NA	X07652	1,543	19
<i>Escherichia coli</i>	NA	J01695	1,542	19
<i>Vibrio anguillarum</i>	NCMB 6	X16895	1,503	20
<i>Vibrio harveyi</i>	ATCC 14126	X56578	1,484	21
<i>Vibrio campbelli</i>	ATCC 25920	X56575	1,484	21
<i>Vibrio alginolyticus</i>	ATCC 17749	X56576	1,482	21
<i>Vibrio diazotrophicus</i>	ATCC 33466	X56577	1,464	21
<i>Vibrio parahaemolyticus</i>	ATCC 17802	X56580	1,499	21
<i>Vibrio vulnificus</i>	ATCC 27562	X56582	1,467	21
<i>Vibrio hollisae</i>	ATCC 33564	X56583	1,476	21
<i>Vibrio cholerae</i>	ATCC 14035	Z21856	1,341	R. Rosson
<i>Vibrio orientalis</i>	ATCC 33933	Z21731	1,363	R. Rosson and K. Nealson
<i>Photobacterium leiognathi</i> PL721	NA	Z21730	1,285	R. Rosson and K. Nealson
<i>Photobacterium fischeri</i> MJ-1	<i>Monocentris japonica</i>	Z21729	1,290	R. Rosson and K. Nealson
<i>Photobacterium phosphoreum</i>	<i>Opisthoproctus grimaldii</i>	Z19107	1,460	16
Unknown	<i>Melanocetus johnsoni</i>	Z19105	1,420	16
Unknown	<i>Cryptosaras couesi</i>	Z19106	1,416	16
Unknown	<i>Kryptophanaron alfredi</i>	Z19003	1,429	This study
Unknown	<i>Anomalops katoptron</i>	Z19081	1,461	This study
Unknown	<i>Photoblepharon palpebratus</i> #1	Z19085	1,416	This study
Unknown	<i>Photoblepharon palpebratus</i> #2	Z19079	1,266	This study
Unknown	<i>Photoblepharon steinetzi</i>	Z19080	1,390	This study

any of the taxa for which sequences are available and do not fall within the genus *Photobacterium* (Fig. 2). The anomalopid symbionts form one monophyletic group and the ceratioid symbionts another. Differences among the symbionts from different ceratioid families are as large as the differences among species of *Vibrio*. Likewise, the symbionts from different genera of anomalopids diverge deeply. These results are in distinct contrast to the findings from *P. phosphoreum*; three symbiotic strains from two different host orders were indistinguishable at all positions for which 16S rRNA sequence was obtained (ref. 16 and unpublished data). This system is also quite different from the coral zooxanthellae symbiosis in which the symbionts were found to be relatively genetically undifferentiated among a wide taxonomic range of hosts¹⁷, but is similar to that of intracellular chemoautotrophic bacterial symbionts¹⁸.

Neither the extent to which the anomalopid symbionts have evolved in parallel with their hosts, nor the point in the history

of each of these symbioses at which this parallelism might have begun can yet be determined definitively. The *Anomalops* symbiont differs greatly from the *Photoblepharon palpebratus* symbionts, even though the fish were collected in the same location (Fig. 3). *Photoblepharon* symbionts from different locations (*P. steinetzi*, Red Sea and *P. palpebratus*, Papua New Guinea) group together. Similar results were obtained in a previous restriction fragment length polymorphism (RFLP) study¹⁵. The differences between symbiont sequences from different host genera in spite of overlap in the geographic occurrence of those host genera suggests a specific association in which the symbionts may have diverged in parallel with their hosts. But the topology of the anomalopid symbiont 16S rRNA tree, although robust, is in conflict with the currently accepted phylogeny of the host family². The *Kryptophanaron* symbiont diverges most deeply in the 16S rRNA gene tree, but the genus *Anomalops* is proposed to be the most ancient divergence in the family Anomalopidae.

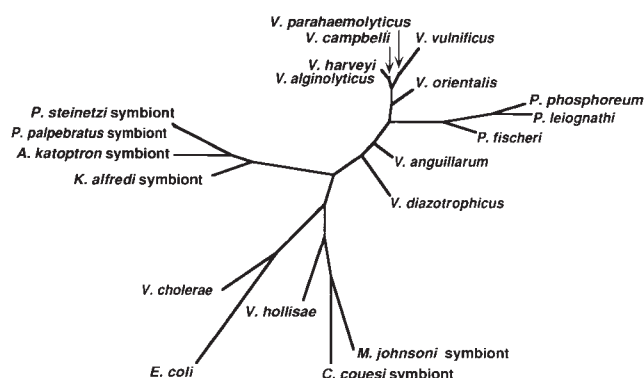


FIG. 2 Parsimony analysis of *Vibrio* spp. and relatives produced with PAUP. The dataset included 1,195 positions. *E. coli* was the outgroup. The heuristic search algorithm was repeated 500 times with starting trees constructed by random addition. Two minimal trees were found that differed within the anomalopid symbiont group. The tree shown is that which agrees with the analysis shown in Fig. 3.

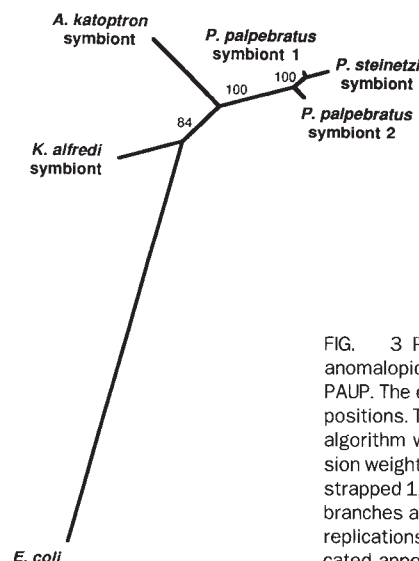


FIG. 3 Parsimony analysis of anomalopid symbionts produced with PAUP. The dataset used included 1,190 positions. The branch and bound search algorithm was used with the transversion weight of 5. The dataset was bootstrapped 1,000 times. Numbers beside branches are the per cent of bootstrap replications in which the group indicated appeared in the shortest tree.

Possible explanations are: (1) the host phylogeny is in error; (2) the *Kryptophanon* symbiont became obligate and was isolated from free-living populations earlier than the *Anomalops* and *Photoblepharon* symbionts; or (3) the symbiont of either *Anomalops* or *Photoblepharon* displaced the other when the two host genera became sympatric, and subsequently diverged along with their hosts. A molecular phylogeny of the host family may help to clarify the issue. Another surprising observation is that symbionts of two specimens of *P. palpebratus* are quite distinct (Fig. 3). In contrast, six *K. alfredi* from two locations in the Caribbean and *A. katoptron* from two locations were identical within their respective host genera¹⁵. The *P. palpebratus* samples came from two morphological types from the same location, and may reflect an unrecognized taxonomic difference in the hosts.

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Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells

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ACTIVATION of CD4⁺ T helper cells results from the occupancy of the T-cell receptor (TCR) by immunogenic peptide bound to a class II major histocompatibility complex (MHC) molecule¹, together with a co-stimulatory signal from the antigen-presenting cell (APC)². This activation leads to proliferation, cytokine production (Th1 or Th2 profile) and cytotoxicity³. Engagement of the TCR in the absence of co-stimulation causes Th1 cells to become unresponsive to subsequent antigenic stimulation^{4–6}. We have previously demonstrated that analogues of an immunogenic peptide could stimulate Th1 and Th2 cells to carry out some effector functions without inducing proliferation^{7,25}, a phenomenon we term partial activation. Here we study the consequences of such partial activation through the TCR of two Th1 clones using peptide analogues presented by a live APC. A peptide analogue that is unable to stimulate clonal proliferation or production of cytokine or inositol phosphate can induce the T cells to become profoundly unresponsive to subsequent stimulation with the immunogenic peptide. Thus, altering the ligand of the TCR by using a peptide analogue on a functional APC sends a signal to Th1 clones that results in anergy.

The Th1 clone PL.17 is specific for the immunogenic peptide of the murine haemoglobin β^d minor chain, Hb β (64–76), bound to an I-E^k molecule. Peptide analogues were constructed by introducing conservative, single amino-acid substitutions at the previously identified TCR contact positions of this peptide⁸. Using an I-E^k-transfected fibroblast line, DCEK Hi7 (ref. 9), as APC, we analysed the functional responses of PL.17 cells after stimulation with the immunogenic peptide or analogues containing substitutions of serine for alanine at position 70 (Ser 70), or of glutamine for asparagine at position 72 (Gln 72). These analogues bind to I-E^k molecules with an affinity compar-

able to the Hb(64–76) peptide, as evidenced by their effective competition with ¹²⁵I-labelled Hb(64–76) in a direct binding assay⁸. The Ser-70 and Gln-72 analogues did not induce T-cell proliferation, or production of Th1 cytokines (interleukins IL-2 and IL-3, and interferon- γ) or free inositol phosphates (Fig. 1; IL-3 data not shown). Parallel experiments using B10.BR (Hb β^s) spleen cells as APC gave similar results.

Upon activation, T cells upregulate various cell-surface molecules, including the IL-2 receptor and lymphocyte function antigen LFA-1 (refs 10, 11). Flow cytometric analysis revealed that the Ser-70 analogue, like the immunogenic peptide, stimulated upregulation of both the IL-2 receptor (Fig. 2a) and LFA-1 (Fig. 2b). Surface expression of other molecules, including CD3 and class I major histocompatibility complex (MHC), did not change. Stimulation with the Gln-72 analogue did not generally lead to upregulation of the IL-2 receptor or LFA-1, although each surface molecule was increased slightly in two of six experiments. Consistent with T-cell stimulation⁵, PL.17 cells activated with the Ser-70 analogue, but not the Gln-72 analogue, showed a significant cell volume increase by flow cytometric analysis (data not shown). Thus, the Ser-70 analogue was delivering a signal to PL.17 upon TCR engagement which allowed upregulation of IL-2 receptors and of LFA-1, and increased cell volume, but did not activate cytokine production or proliferation.

These interesting properties of the Th1 clone PL.17 and the Ser-70 analogue peptide enabled us to evaluate whether TCR engagement without proliferation leads to anergy¹². We used a proliferation assay to study PL.17 previously stimulated with DCEK Hi7 or B10.BR spleen cells as a source of live APC, either alone or with peptide analogues. PL.17 cells cultured with APC alone, or APC and the Gln-72 analogue, proliferated vigorously upon subsequent stimulation with Hb(64–76). But PL.17 cells first cultured with APC and the Ser-70 analogue were now completely unresponsive to the immunogenic peptide (Fig. 3a, b). These PL.17 cells were tolerant to stimulation with Hb(64–76), but the proliferation response was normal to exogenous IL-2 (Fig. 3 legend) or to phorbol myristate acetate and Ca²⁺ ionophore (data not shown). When B10.BR lipopoly-saccharide-treated spleen cell blasts and B-cell lymphomas were used as sources of live APC, we still found Ser-70 analogue-induced anergy of PL.17. There were two other important results. First, preincubation of another Hb(64–76)-specific Th1 clone, Hb#2, with the Ser-70 analogue also resulted in unresponsiveness

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to the immunogenic peptide (Fig. 3c). However, the Ser-70 analogue stimulated a cytolytic response, but not a proliferative response, from Hb#2 cells²⁵, an indication that this peptide analogue caused partial activation of this T-cell clone. Second, the Ser-70 analogue is capable of causing proliferation of other haemoglobin-specific T-cell clones (2 of 6 clones)⁸. This indicates that the interaction between TCR and the peptide-MHC complex, and not the peptide itself, is what determines the subsequent T-cell response. We are now investigating T-cell receptor usage among these different clones to see whether there is any correlation with the anergic/stimulatory response.

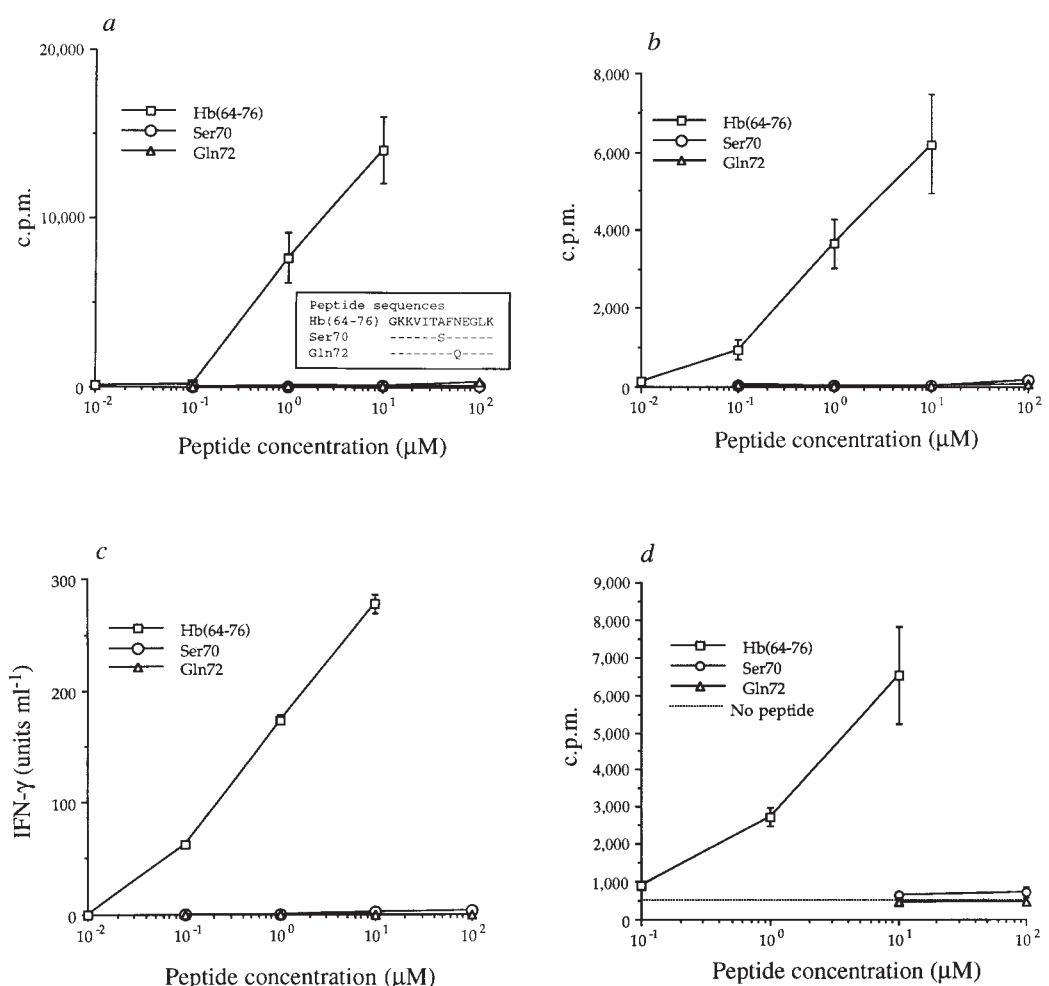
We showed that this tolerance of T cells was not due to carryover of APC presenting Ser-70 analogue or to temporary refractoriness of the T cells in the following experiments. To eliminate the effect of analogue carryover, graded doses of APC preincubated with the Ser-70 analog (100 μ M) were added simultaneously with fresh APC and Hb(64-76), and there was

no inhibition of proliferative response of PL.17 (<5% of the response). To see whether there was temporary refractoriness of T cells after antigen stimulation^{13,14}, we incubated PL-17 cells with either a proliferative dose of Hb(64-76) (10 μ M) or a tolerogenic dose of Ser-70 analogue (50 μ M) and compared their responses to immunogenic peptide after 7 days. As the T cells could mount a proliferative response after incubation with Hb(64-76) (58,974 c.p.m.) but not after incubation with the Ser-70 analogue (10,039 c.p.m.), they were not refractory to antigen restimulation at this time point.

Previous studies using chemically fixed spleen cells and immunogenic peptide indicated that the unresponsive state of T cells lasted for a relatively long time (tested up to 8 days)⁴. Addition of cyclosporin A (CsA) or an exogenous source of costimulation to the culture prevented the T cells from becoming anergic^{15,16}. Similarly, the Ser-70 analogue also induced long-lasting anergy in PL.17 because the T cells were still unresponsive

1 Proliferation, lymphokine production and inositol phosphate generation responses of PL.17 clone to Hb(64-76) and its analogue peptides. *a*, Proliferation response; *b*, production of IL-2 and, *c*, interferon (IFN)- γ ; and *d*, inositol phosphate generation induced by Hb(64-76), and its Ser-70 and Gln-72 peptides.

METHODS. Proliferation was assayed in 96-well flat-bottomed plates in 200 μ l RPMI-1640 medium containing 10% fetal calf serum (Hyclone), 2 mM glutamine, 50 μ g ml⁻¹ gentamycin, 10 mM HEPES and 2-mercaptoethanol (2×10^{-5} M). The following were added to the appropriate wells: PL.17 clone, at 2×10^4 cells per well, mitomycin C-treated DCEK Hi7 fibroblasts (77μ g ml⁻¹) in Hank's balanced salt solution for 90 min at 37 °C (Sigma) transfected with I-E^k construct⁹ or CH27 B lymphoma cells (for IL-2 assay) at 5×10^4 cells per well, and Hb peptides (0–100 μ M). The assay was incubated at 37 °C for 72 h with addition of ³H-thymidine (0.4 μ Ci per well) during the last 20 h. Lymphokine responses were assessed using 24-h (IL-2) or 48 h (IFN- γ) supernatants from these cultures. IL-2 was quantitated in a bioassay as proliferation of the IL-2-dependent cell line CTLL-2 (ref. 21) as described²². IFN- γ was measured by ELISA using reagents provided by R. Schreiber²³. Monoclonal antibody H22 was used to capture IFN- γ from test supernatants, and bound cytokine was identified with a polyvalent rabbit anti-murine IFN- γ , followed by peroxidase-conjugated goat anti-rabbit IgG (TAGO). The substrate was developed with ABTS (2,2'-azino-di-[3-ethyl-benzthiazolinsulphonate(b)]) reagent and the absorbance read at 414 nm. For inositol phosphate detection, PL.17 cells were incubated overnight at $1-2 \times 10^7$ cells per ml in inositol-free RPMI complete medium containing 20–50 μ Ci ml⁻¹ myo-[2-³H]inositol (Amersham). Cells were then washed in HBSS and resuspended in medium with 10 mM LiCl (inositol phosphatase inhibitor). T cells were incubated in 96-well flat-bottomed plates ($7-10 \times 10^5$ /well) with the indicated doses of Hb(64-76) or peptide analogue and DCEK Hi7 ($7-10 \times 10^5$ per well) for 90–120 min and assayed for accumulation of free inositol phosphates as described²⁴. Briefly, cultures were extracted with 1 ml of a 1/2 mixture of chloroform and methanol followed by 0.25 ml chloroform and H₂O. Phases were separated by centrifugation and the H₂O-soluble



fraction run on a 0.25 ml AG1-X8 formate ion-exchange column (BioRad), then washed extensively with 5 mM myo-inositol. Total free inositol phosphate was eluted from the column with 0.1 M formic acid, 1 M sodium formate in 1.5 ml, and the radiolabel quantitated by scintillation counting. IL-3 was quantitated using a bioassay²⁵. For some proliferation and lymphokine assays, DCEK Hi7 cells were substituted by irradiated B10.BR/SgSnJ spleen cells (2,000 rads, 5×10^5 cells per well). The Hb(64-76) peptide and peptide analogues were synthesized using a DuPont RaMPS apparatus, purified by reverse-phase HPLC, analysed and quantitated using a Beckman 6300 amino-acid analyser. The sequence of the Hb(64-76) peptide and the analogues substitutions are shown in the insert to *a*. Experiments were done in triplicate, or in duplicate for inositol phosphate generation; error bars represent standard deviation of the mean. Each experiment is representative of 4–5 independent assays.

to the immunogenic peptide seven days after culture with this analogue (Fig. 4a). CsA addition with the Ser-70 analogue prevented induction of anergy (Fig. 4b). But, in contrast to the anergy induced on chemically fixed APC, in this system addition of allogeneic spleen cells depleted of T lymphocytes as a source of co-stimulation had no effect (data not shown). Furthermore,

anergy of PL.17 was not induced by presentation of the Ser-70 analogue on chemically fixed APC. These observations suggest that anergy induced by stimulating peptides with fixed APC is different from that induced by analogues with live APC.

Our finding that T cell anergy can occur by presentation of peptide analogues on a live functional APC is not the same

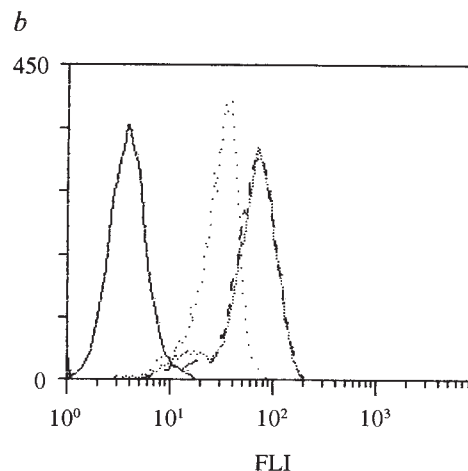
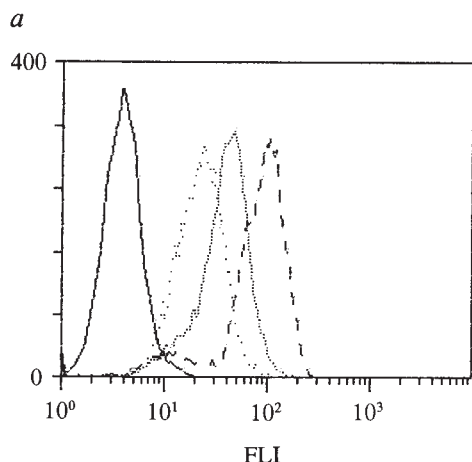


FIG. 2 FACS analysis reveals that the Ser-70 analogue induces upregulation of *a*, the IL-2 receptor and *b*, LFA-1 on PL.17; x-axis represents fluorescence intensity (FLI) and Y-axis represents relative cell number.

METHODS. PL.17 (5×10^5 cells per well) were incubated with DCEK Hi7 L cells (5×10^5 per well) alone (—) or with 50 μ M Ser70 analogue (···) or 0.01 μ M Hb(64-76) (---) in 24-well plates for 48 h. T cells were then separated from L cells by centrifugation over Ficoll-Hypaque (Pharmacia) at

3,000 r.p.m. for 15 min, and washed 3 times before FACS analysis. For each group, 1×10^5 cells were incubated with anti-IL-2 receptor (*a*; 7D4, rat IgG) or anti-LFA-1 (*b*; FD441.8, rat IgG), and captured using a fluorescein isothiocyanate-labelled goat anti-rat IgG (Southern Biotech). PL.17 cells, preincubated with DCEK Hi7 cells alone, were stained with goat anti-rat IgG alone as a negative control (—). Cells were analysed on a FACScan (Becton Dickinson). Data are representative of 4 different experiments.

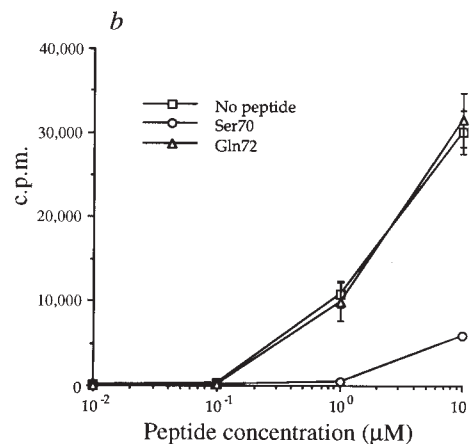
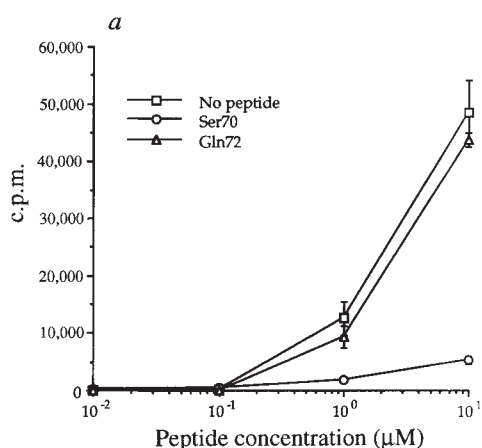
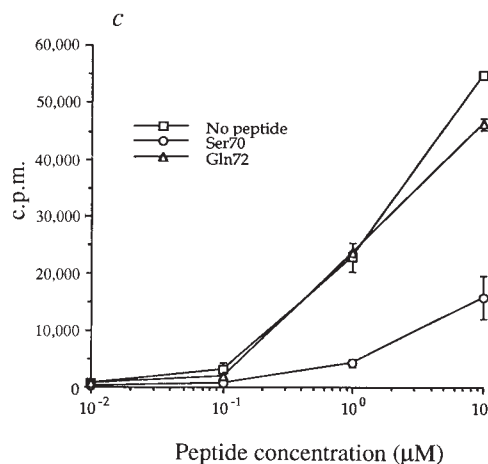


FIG. 3 Ser-70 analogue induces anergy of PL.17 and Hb2. Th1 cells (5×10^5 per well) and mitomycin C-treated APC were incubated alone or with 50 μ M Ser-70 analogue or 50–100 μ M Gln-72 analogue for 20–24 h at 37 °C in 24-well tissue culture plates in a final volume of 700 μ l. T cells were then separated from APC as for Fig. 2, and rested for 2–3 days in 48-well tissue culture plates in RPMI medium before challenge in a proliferation assay (Fig. 1 legend) using Hb(64-76) as antigen, or with IL-2 (20 units per well). T cells and APC were used as follows: *a*, PL.17 Th1 cells and DCEK Hi7 L cells (5×10^5 per well); *b*, PL.17 Th1 cells and B10.BR/SgSnJ spleen cells (5×10^6 per well); *c*, Hb2 Th1 cells and DCEK Hi7 L cells. Cell recovery after the rest period, expressed as a percentage of input cell number, was: *a*, 50% for all three groups; *b*, 60% for no-peptide and Gln-72 groups, and 50% for Ser-70 group; *c*, 75% for no-peptide group, 90% for Ser-70 group, and 83% for Gln-72 group. IL-2 responses were: *a*, no peptide, $12,270 \pm 2,493$ c.p.m.; Ser-70 Hb(64-76), $15,751 \pm 689$ c.p.m., and Gln-72 Hb(64-76), $10,864 \pm 843$ c.p.m. *b*, No peptide, $18,061 \pm 6,468$ c.p.m.; Ser-70 Hb(64-76), $29,447 \pm 5,738$ c.p.m., and Gln-72 Hb(64-76), $11,816 \pm 2,365$ c.p.m. *c*, No peptide, $70,543 \pm 4,009$ c.p.m.; Ser-70 Hb(64-76), $66,654 \pm 6,766$ c.p.m., and Gln-72 Hb(64-76), $50,929 \pm 3,857$ c.p.m. In some cases, DCEK Hi7 L cells or CH27 cells (5×10^4) replaced the spleen in the challenge assay, giving similar results. Data points represent the mean of triplicate cultures $\pm$ s.d. Each experiment is a representative from 3 (*b*), 4 (*c*) or 9 (*a*) independent assays.



phenomenon as a previously described peptide antagonism that has been explained in terms of a stochastic model of competition for TCR sites by analogue peptides. In this system¹⁷, inhibitory antigen analogues cannot induce T cell tolerance; in our system, competition for TCR sites is not implicated in the lack of response because the analogue peptide is removed before stimulation with immunogenic peptide. A peptide analogue may interact with lower affinity with the TCR, so only part of the normal signal is delivered upon TCR engagement. This could result because of rapid dissociation of the TCR from the peptide-MHC complex, or a lack of allosteric changes in the TCR, or because there is no association of surface regulatory molecules with the TCR, or combinations of these factors. This partial signal, in the absence of a full stimulatory signal, results in T-cell anergy rather than clonal expansion. As the TCR/CD3 complex has multiple chains that are thought to be involved in signal transduction upon ligand interaction, some, but not all, of these chains may be engaged upon presentation of anergy-inducing peptide analogues. Indeed, some chains of the CD3 complex can transduce signals independently of the others¹⁸⁻²⁰. These results were not peculiar to this particular peptide analogue, because preincubation with the Asp-73 analogue, which also fails to stimulate PL.17 proliferation, inhibits the proliferative response to Hb(64-76) (77% inhibition). We have observed a similar pattern of reactivity for several T-cell clones.

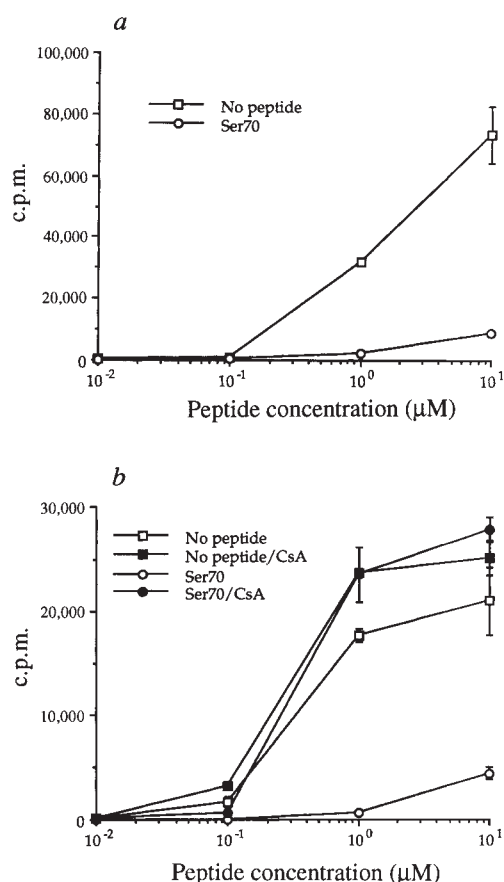


FIG. 4 Ser-70 analogue-induced energy is *a*, long-term and *b*, prevented by cyclosporin A.

METHODS. PL.17 cells were used in the tolerance assay with DCEK Hi7 L cells as described for Fig. 3a, with the addition of cyclosporin A (1 μg ml⁻¹) in *b*. T cells were separated from APC as described in Fig. 2 legend and were then rested in 48-well tissue culture plates for *a*, 7 days, or *b*, 3 days, before being used in the challenge assay described in Fig. 3 legend. Data points represent means of triplicate cultures ±s.d. Each experiment is representative of 5 (*a*) or 3 (*b*) independent assays.

(J.S.L., unpublished observations). Our results show that, by introducing conservative amino-acid substitutions in the TCR contact residues of an immunogenic peptide, the interaction of the ligand can be changed so that only part of the signal is delivered. The extent of the signal transduced appears to be dependent on which TCR contact residue is altered, with changes in more permissive residues inducing T-cell anergy, and changes in less permissive residues preventing the delivery of any signal to the T cell. Most ligands for T cells should be amenable to such manipulation, an approach that may eventually have clinical application.

Note added in proof: In another system, evidence for ligand-related differences in T-cell receptor-dependent intracellular signalling was recently demonstrated by peptide-MHC class II complexes with mixed agonist/antagonist properties²⁶. □

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Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide

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SEVERAL observations suggest interactions between the immune and nervous systems^{1,2}. Psoriasis and atopic dermatitis may worsen with anxiety and have been associated with anomalous neuropeptide regulation². Neurotransmitters affect lymphocyte function¹⁻⁴ and lymphoid organs are innervated⁵⁻⁹. Calcitonin gene-related peptide (CGRP) is a neuropeptide and vasodilator¹⁰ that modulates some macrophage functions, including antigen presentation *in vitro*¹¹. CGRP is associated with Langerhans cells (LC) in oesophageal mucosa, particularly during inflammation¹², is present in epidermal nerves and is associated with Merkel cells^{10,13-15}. We

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examined the ability of CGRP to modulate LC antigen-presenting function¹⁶ and asked if CGRP-containing nerves impinge on LC. We report here that CGRP-containing nerve fibres are intimately associated with LC in human epidermis and CGRP is found at the surface of some LC. In three functional assays CGRP inhibited LC antigen presentation. These findings indicate that CGRP may have immunomodulatory effects *in vivo* and suggest a locus of interaction between the nervous system and immunological function.

Double immunofluorescence staining was done on normal adult human skin using antibodies to CGRP and CD1a to identify CGRP-containing axons and LC. A network of highly dendritic LC was observed¹⁷ and the distribution of CGRP⁺ nerve fibres was similar to that previously described^{14,18}. Most axons had a beaded staining pattern and coursed through the superficial dermis and epidermis in a serpiginous fashion (Fig. 1a). They appeared as both single and branched processes, often having a 'Y' -shaped bifurcation of the intraepidermal com-

ponent.

Confocal laser microscopy showed intimate associations of CD1⁺ epidermal LC with CGRP⁺ epidermal nerves, in contrast to previous reports suggesting that CGRP⁺ intraepidermal axons represent 'free nerve endings'¹⁴⁻¹⁸. Apparent contacts between the two were documented in 70-80% of CD1⁺ epidermal LC when focusing through 30- μ m-thick tissue sections (Fig. 1b, c). Nerves consistently appeared to terminate at LC bodies and association with LC dendrites was not observed. Some CGRP⁺ axons originate in the dermis, transgress the basement membrane zone, and enter the epidermis where they were consistently aligned towards CD1⁺ LC. Occasional LC bodies showed patchy reactivity for CGRP (Fig. 1d, e), consistent with deposition by nearby axons which approximated cell bodies but not LC dendrites. These observations were confirmed at the ultrastructural level as shown in Fig. 1f, where an LC was visualized with an axon-like process impinging directly on it.

We examined the effect of CGRP on alloantigen presentation

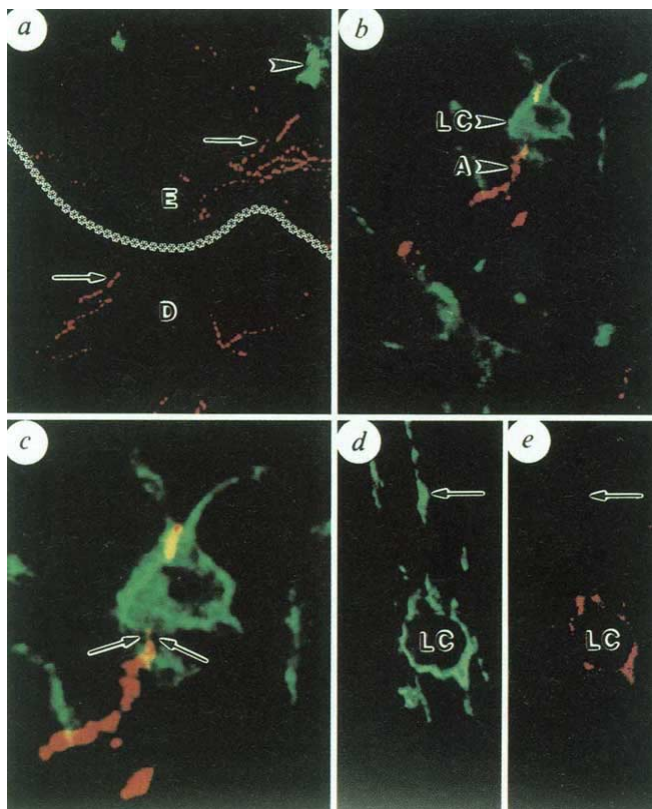


FIG. 1 a-e, Confocal laser scanning microscopy of CD1⁺ and CGRP⁺ cells in human skin. a, Branching plexuses of CGRP⁺ axons (arrows) in the dermis (D) and epidermis (E) (dermal-epidermal junction demarcated by asterisks). The axons depicted by the arrows were contiguous in sequential optical sections and approximated a CD1⁺ LC within the epidermis (arrowheads). b, Apparent contact between a CGRP⁺ axon (A) and an epidermal LC; c, This association (arrows) at higher magnification. The red-yellow zone at the superior pole of the cell presumably represents an axon in tangential section. d, e, Single-channel analyses of LC CD1 reactivity (d) and patchy CGRP staining along the membrane of the LC body (e). This pattern involved a minority (<10%) of LC, did not involve CD1⁺ dendrites (arrows), and is consistent with CGRP released from axons that consistently approximated cell bodies but not dendrites. f, Transmission electron micrograph (TEM) of axon-like structure (arrow and lowermost inset) in apposition to a LC. The axon-like structure, as well as cross-sections through underlying superficial dermal unmyelinated axons (arrows, uppermost inset), are composed of membrane-bound cylinders of lucent cytoplasm, corresponding to axons, enveloped by cell extensions containing darker cytoplasm, corresponding to Schwann cell processes. The adjacent LC contains granules (enclosed by small rectangle and denoted in middle inset by an asterisk) that permit specific identification of this cell type.

METHODS. Specimens of normal adult human skin were snap frozen in OCT



(Miles), and stored at -70 °C until use. Serial frozen sections (30 μ m) were prepared as described²⁷, incubated with rabbit anti-human CGRP antiserum (1:50; Peninsula Labs) for 90 min at room temperature and rinsed. Mouse anti-human CD1a (1:50; Becton Dickinson) was applied to the sections for 90 min followed by rinsing. FITC-conjugated sheep anti-mouse immunoglobulin (1:25; Accurate Chemical) was applied for 60 min to label CD1. Texas red-conjugated donkey anti-rabbit immunoglobulin (dilution 1:25; Accurate Chemical) was applied for 60 min to label CGRP. Controls consisted of substitution of primary antibodies with isotype-specific irrelevant antisera. Sections were viewed with an MRC-600 Confocal Imaging System (Bio-Rad Microscience) as previously described²⁷. For TEM, normal adult human skin was processed and sections stained with uranyl acetate and bismuth subnitrate as previously described²⁸. Sections were examined using a Hitachi 7000 transmission electron microscope.

by freshly obtained murine epidermal cell (EC) populations to allogeneic T lymphocytes in the mixed epidermal cell/lymphocyte reaction (MELR)¹⁹. Pretreatment of EC with 10 nM or 100 nM CGRP inhibited the MELR response, whereas pretreatment of responding lymphocytes did not (Fig. 2), suggesting that CGRP acts on the stimulating cells.

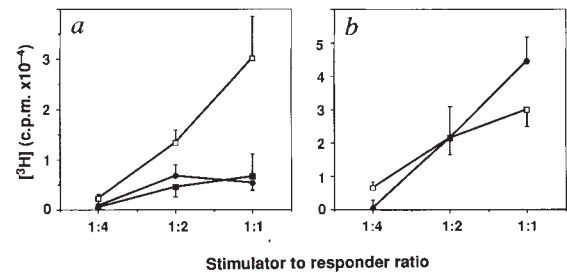
CGRP also inhibited EC presentation of a defined protein, chicken ovalbumin (cOVA), to the cOVA responsive line DO11.1 (ref. 20; Fig. 3). Response of DO11.1 cells to a fragment of

cOVA which does not require processing²⁰ was also inhibited.

To define the effect of CGRP on EC antigen presentation further, a mixed *in vitro/in vivo* system was used in which EC exposed to CGRP and pulsed with soluble tumour-associated antigens (TAA) *in vitro* are used to elicit a delayed-type hypersensitivity (DTH) response *in vivo* in tumour-immune mice²¹. Deletion of Ia⁺ cells (Langerhans cells) eliminated the ability of EC to elicit immunity in this system (Fig. 4a). Pre-exposure of EC to CGRP for 2 h before TAA pulsing resulted in a

FIG. 2 Preincubation of EC in CGRP inhibits their ability to present alloantigen in the MELR. *a*, EC preincubated in medium alone (positive control) (empty squares); EC preincubated in 10 nM (filled squares) or 100 nM CGRP (filled circles). 1 nM CGRP produced little or no inhibition of the response. *b*, No pretreatment of EC but T cells preincubated in medium alone (empty squares) or 100 nM rat CGRP (closed circles). Bars, s.e.m. Background proliferation of EC or T cells alone was <1,000 c.p.m. TGF- β 2 (1–10 ng ml⁻¹) failed to inhibit EC stimulation in this system, whereas a CGRP fragment (CGRP_{8–37}; Peninsula Labs) demonstrated some inhibitory activity at 10–100 nM in some experiments (not shown).

METHODS. EC generated from BALB/c mice as described²¹ were incubated in 1 nM, 10 nM, or 100 nM rat CGRP (Peninsula); or medium alone for 3 h. Cells were washed 3 times and plated in triplicate in round-bottom 96-well plates together with 2×10^5 nylon wool-purified allogeneic T cells (C3H/HeJ mice). Cells were incubated for 6 days in RPMI 1640 medium containing 1.5% heat-inactivated mouse serum, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 μ M HEPES, 50 μ M 2-mercaptoethanol and 5 μ g ml⁻¹ indomethacin, and pulsed for an additional 24 h with 1 μ Ci per well [³H]thymidine. In a separate experiment, allogeneic T cells were preincubated with 100 nM CGRP for 3 h before plating together with various numbers of untreated EC. Deletion of Ia⁺ cells by antibody and complement abrogates



the ability of EC to stimulate in the MELR (ref. 19, and data not shown). Because of the possibility that CGRP might be toxic to LC, I-A reactivity in EC exposed to 1 nM, 10 nM or 100 nM CGRP, or medium alone was determined by FACS analysis after 3 h exposure. No differences were seen in the number of cells expressing I-A. EC treated with medium alone or 100 nM CGRP were also examined at 24 and 48 h. No difference in the number of cells expressing I-A was observed, although the proportion of I-A⁺ cells was reduced in both groups with increasing time. The intensity of I-A expression was unchanged with 1 and 10 nM CGRP, but was slightly reduced in some experiments at 100 nM CGRP (not shown).

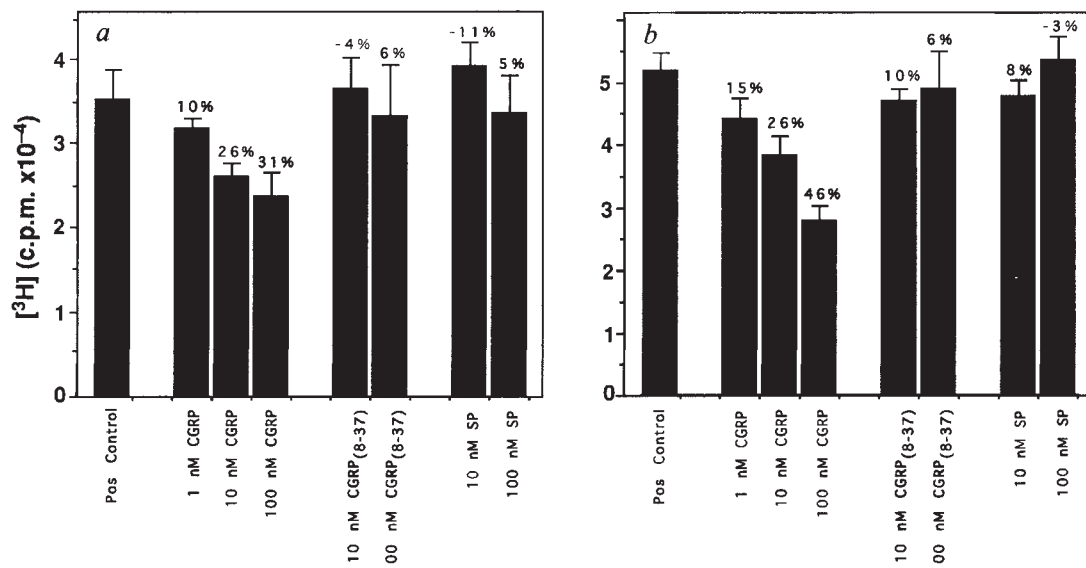


FIG. 3 *a*, Incubation in CGRP inhibits the ability of EC to present cOVA to the T-T hybridoma DO11.1. A representative experiment demonstrates inhibition of presentation by CGRP in a dose-dependent manner from 1–100 nM. The control peptides CGRP_{8–37} and substance P (SP; Peninsula) failed to demonstrate significant inhibition. The ordinate represents c.p.m. of each group minus background c.p.m. (negative control) $\pm$ s.e.m. The per cent inhibition compared to the positive control is shown above each column. Positive control versus 10 nM CGRP, $P=0.039$; versus 100 nM CGRP, $P=0.036$; versus 1 nM CGRP, 10 nM CGRP_{8–37}, 100 nM CGRP_{8–37}, 10 nM SP and 100 nM SP, not significant (two-tailed Student's t -test). *b*, CGRP inhibited presentation of a recognized fragment of cOVA to DO11.1 cells. A representative experiment demonstrates inhibition by CGRP in a dose-dependent fashion in the range of 1–100 nM. CGRP_{8–37} and SP failed to inhibit the response observed. Positive control versus 10 nM CGRP, $P=0.014$; versus 100 nM CGRP, $P<0.001$; versus 1 nM CGRP, 10 nM CGRP_{8–37}, 100 nM CGRP_{8–37}, 10 nM SP and 100 nM SP, not significant.

METHODS. EC from BALB/c mice were incubated in CGRP, 1.5 nM, 15 nM, 150 nM, 15 nM CGRP_{8–37}, 150 nM CGRP_{8–37}, 15 nM SP, 150 nM SP or medium alone for 2 h followed by addition of cOVA (Sigma) to a final concentration of 333 μ g ml⁻¹ or a recognized heptadecapeptide fragment²⁰ (J. Greenstein, ImmunoLogic) to final concentration of 0.33 μ M. Antigen added in PBS resulted in dilution of the test substance to 1 nM, 10 nM and 100 nM, as indicated. Incubation was continued at 37 °C for 18 h. Cells (10^5) were washed 3 times and plated in sextuplet in flat-bottomed 96-well plates, together with 10^5 DO11.1 cells in 200 μ l RPMI 1640 containing 10% heat-inactivated FCS, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 50 μ g ml⁻¹ gentamicin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate and 10 μ M HEPES buffer. Negative control wells contained EC not exposed to antigen (PBS alone) with DO11.1 cells. After incubation at 37 °C for 24 h, 100 μ l of supernatant was examined for interleukin-2 (IL-2) content by ability to support the proliferation of the IL-2/IL-4-dependent cell line CTLL.

dose-dependent inhibition of the ability to elicit DTH (Fig. 4b). The inhibition was not due to carryover of CGRP into the host animal, because injection of EC pulsed with antigen, together with EC not pulsed with antigen but preincubated in CGRP, allowed for a full response (Fig. 4c). This mixing experiment

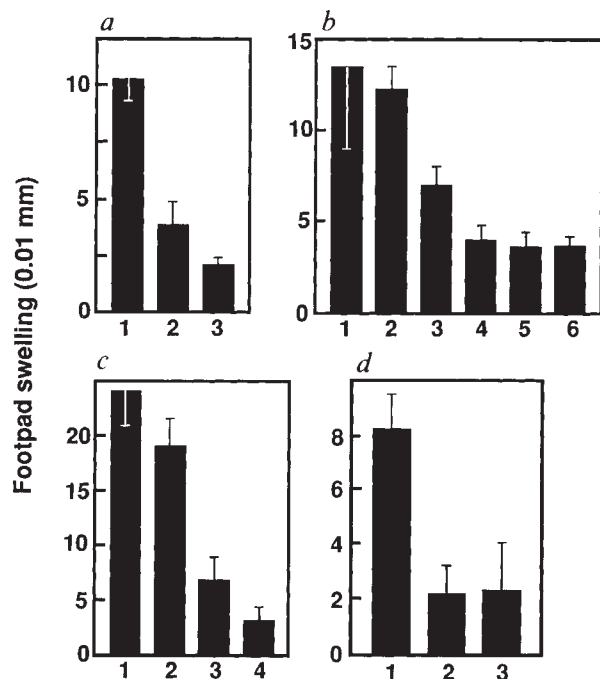


FIG. 4 Exposure of EC to CGRP inhibits elicitation of DTH. **a**, EC pulsed with TAA (1), EC deleted of Ia⁺ cells before TAA exposure (2), EC not exposed to TAA (negative control) (3). 1 versus 2, $P=0.002$; 1 versus 3, $P<0.001$ (two-tailed Student's *t*-test). **b**, EC precultured in rat CGRP (2×10^6 cells per ml) for 2 h at 37 °C before washing and TAA exposure; no CGRP (1), 10 pM CGRP (2), 100 pM CGRP (3), 1 nM CGRP (4), 10 nM CGRP (5), EC not exposed to TAA (6). 1 versus 2, not significant; 1 versus 3, $P=0.012$; 1 versus 4, $P=0.001$; 1 versus 5, $P=0.001$; 1 versus 6, $P=0.001$. **c**, EC (2.5×10^5) preincubated or not preincubated with 10 nM CGRP were mixed with EC (2.5×10^5) not exposed to CGRP but pulsed with TAA for footpad challenge. Cells not treated with CGRP or TAA plus cells treated only with TAA (1), cells treated with CGRP but not exposed to TAA plus cells exposed to TAA only (2), cells preincubated with CGRP and then exposed to TAA plus cells not exposed to CGRP or TAA (3), 5×10^5 EC not exposed to CGRP or TAA (4); 1 versus 2, not significant; 1 versus 3, $P=0.001$; 1 versus 4, $P=0.001$. **d**, Inhibition of the elicitation of DTH by CGRP with EC enriched for LC by FACS. LC not treated with CGRP but pulsed with TAA (1), LC treated with 100 nM CGRP and pulsed with TAA (2), LC not exposed to TAA (3). 1 versus 2, $P=0.002$; 1 versus 3, $P=0.006$. Five mice per group; bars, s.e.m. TGF β 2 (10 ng ml⁻¹), TNF- α (100 U ml⁻¹), and CGRP₈₋₃₇ (100 nM) failed to inhibit EC elicitation of DTH (not shown).

METHODS. CAF1/J mice (H-2^{a/d}) were immunized to the A/J (H-2^a) methylcholanthrene-induced cutaneous spindle cell tumour S1509a (ref. 29) as described²¹. CAF1/J EC were prepared, Thy 1⁺ cells deleted by treatment with anti-Thy 1.2 monoclonal antibody (NEI-001, New England Nuclear) and rabbit complement (Cedarlane) as described²¹. Cells were exposed to soluble S1509a TAA and immunized mice were challenged by injection of a hind footpad with 5×10^5 TAA-pulsed EC with 24 h footpad swelling assessed as described²⁹ as a measure of DTH. In a control group, Ia⁺ cells were deleted by treatment with monoclonal anti-I-A^{b,d,k}/I-E^{d,k} antibody (M5/114, Hybritech) at 1:50 for 30 min at 4 °C followed by complement for 45 min at 37 °C before exposure to TAA. For enrichment of LC, CAF1/J EC were suspended in HBSS with 2% FCS containing FITC-conjugated monoclonal anti-I-A^d antibody (Pharmingen) for 30 min on ice, washed and I-A⁺ cells sorted on a FACS (EPICS 750, Coulter); these cells were ~70% I-A⁺. Sorted cells were incubated in 100 nM CGRP or medium alone (2 h), exposed to TAA, washed and injected (5×10^3) into a hind footpad of each mouse.

also suggests that CGRP does not act indirectly by stimulating keratinocytes to produce an inhibitor of LC. To address this possibility further, fluorescence-activated cell sorting (FACS) was used to obtain an enriched population of LC (~70% LC); CGRP also inhibited the ability of these cells to elicit DTH in this system (Fig. 4d). Furthermore, presentation of cOVA by the murine myeloma line A20 to DO11.1 cells is inhibited by CGRP (not shown). Thus CGRP probably acts directly on the antigen-presenting cells (APC). Preliminary experiments indicate that exposure of EC to 100 nM CGRP after pulsing with TAA also leads to an inhibited DTH response (data not shown).

Several control proteins were examined for effects on LC antigen-presenting capability (see legends to Figs 2-4). Transforming growth factor (TGF) β 2, tumour necrosis factor (TNF) α , and a fragment of CGRP (CGRP₈₋₃₇), consistently had no effect in the DTH assay. TGF- β failed to inhibit presentation in the MELR but CGRP₈₋₃₇ showed some inhibitory activity in some experiments. In the DO11.1 system, substance P (see legend to Fig. 3) had no effect, whereas CGRP₈₋₃₇ had slight activity in some experiments. Some activity of CGRP₈₋₃₇ may be expected as it has slight agonist activity in other assay systems²².

These results suggest that CGRP may be a mediator of neurological modulation of LC function and provide an explanation for the demonstration that LC become more potent APC in culture^{23,24}. The CGRP concentrations used in these experiments may be reasonable because concentrations in neuronal tissue as high as 1,197 pg mg⁻¹ (~315 pmol g⁻¹) (lumbar spinal cord) have been reported²⁵ and the effective concentration at local sites of release might be higher. Removal of LC from an environment containing CGRP may account for the increased antigen-presenting function that develops in culture. This hypothesis does not exclude suggestions that the alteration in APC function results from the influence of granulocyte-macrophage colony-stimulating factor produced by keratinocytes contaminating LC cultures²⁴; both may be correct. These data suggest an important locus for interaction between the nervous system and the immune system. In 1868 when Paul Langerhans identified the cells that bear his name by their ability to bind gold chloride, he speculated that they may subserve neural functions²⁶. The involvement of the nervous system and neuron-derived products in modulation of immunocompetent cells may be important in understanding the physiology of the immune system. □

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Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis

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NEUROTRANSMITTER release is triggered by Ca^{2+} ions binding to an unknown Ca^{2+} receptor within presynaptic terminals^{1,2}. Synaptotagmin, a Ca^{2+} -binding protein of synaptic and other secretory vesicles³, has been proposed to mediate vesicle-plasma membrane interactions during neurotransmitter release^{4–9}. Here we test this hypothesis using the giant synapse of the squid *Loligo pealei*, which because of its unusually large size and well established physiology is uniquely suited for dissecting presynaptic events¹⁰. We find that injection of peptides from the C2 domains of synaptotagmin into squid giant presynaptic terminals rapidly and reversibly inhibits neurotransmitter release. Our data are

consistent with these peptides competitively blocking release after synaptic vesicle docking and indicate that Ca^{2+} probably initiates neurotransmitter release by regulating the interaction of synaptotagmin with an acceptor protein.

We isolated synaptotagmin cDNAs from a squid optic lobe cDNA library. The squid synaptotagmin protein sequence as deduced from six overlapping clones (Fig. 1) was highly homologous with synaptotagmins from both human and *Drosophila* (amino-acid identities of 57 and 51 per cent, respectively). Sequence identity was particularly high in the two repeated cytoplasmic domains, C2A and C2B, which are homologous to the C2 regions of protein kinase C (PKC) and phospholipase A2 (ref. 11) and are thought to mediate binding of both phospholipids⁴ and Ca^{2+} (ref. 6) to synaptotagmin. Antibodies generated against fusion proteins containing amino acids 153–403 of squid synaptotagmin recognized a slightly heterogeneous band of relative molecular mass (M_r) 60,000 on western blots; this band was strongly enriched in synaptic vesicle preparations isolated from squid optic lobe (data not shown).

We used the deduced protein sequence to design three peptides modelled after the putative surface-exposed and well-conserved regions of the C2 domains. These peptides were then microinjected into the giant presynaptic terminal in an attempt to interfere with synaptotagmin functions. Microinjection of a 20-amino-acid peptide corresponding to a basic region (residues 312–331) of the squid synaptotagmin C2B domain (Pep20; see Fig. 1) produced a dramatic inhibition of synaptic transmission^{12,13} as revealed by a strong reduction in postsynaptic potential (p.s.p.) amplitude and rate of rise (Fig. 2a). The inhibitory effects of Pep20 developed over a few minutes and reversed with time (Fig. 2a, right, and b). We attribute this reversibility to dilution into the adjacent presynaptic axon, which acts as a diffusion sink^{12,13}. Consistent with this explanation, recovery from injection of large amounts of Pep20 was slow and sometimes not observed at all. This suggests a competitive mechanism of inhibition, in which peptide action depends on diffusion to and from the release sites. Reversibility also excludes a pressure injection artefact, which always yields an irreversible attenuation of release¹³. A 15-amino-acid peptide, Pep15, corresponding to the equivalent sequence span within the C2A domain (Fig. 1), likewise inhibited neurotransmitter release, albeit less than Pep20 (Fig. 2d, and Table 1). Again, the effects of Pep15 were gradual and reversible, provided small

FIG. 1 Alignment of the deduced squid synaptotagmin sequence with the *Drosophila* and human proteins²⁸. The putative transmembrane region is boxed, and the C2A and C2B domains are overlined. Identical amino-acid positions are indicated by dots; gaps introduced to optimize sequence identities are marked by dashes. Amino-acid residues predicted from the open reading frame in front of the first ATG codon are underlined and may indicate that our cDNAs did not cover the full coding region of the squid synaptotagmin mRNA. Bars indicate sequences selected for the synthesis of peptides on the basis of their surface exposure, hydrophilicity and chain flexibility, as predicted by PC Gene Sequence Analysis Software (University of Geneva, Switzerland). The squid synaptotagmin cDNA sequence has been submitted to the EMBL/Genbank data library under the accession number X72386.

METHODS. A squid optic lobe cDNA library (7.2×10^5 PFU) constructed in phage λ gt 10 (insert sizes 600–5,000 bp; provided by J. Battey) was screened with a ^{32}P -labelled (5×10^8 c.p.m. μg^{-1}) rat synaptotagmin cDNA probe (hybridization conditions were 20% (w/v) formamide, 750 mM NaCl, 75 mM Tris-Cl, pH 7.5, 12.5 mM EDTA, 0.1% (w/v) SDS, 5 × Denhardt's solution and 100 $\mu\text{g ml}^{-1}$ herring sperm DNA at 43 °C). The latter was generated on rat brain cDNA by polymerase chain reaction²⁹ using sense and antisense oligonucleotides corresponding to the codons of amino-acid positions 257–263 and 404–410, respectively, of the rat synaptotagmin cDNA⁴. For amplification, 25 cycles at 94 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s, were used. This led to the identification of 120 hybridization-positive clones, of which six served for insert isolation and DNA sequencing³⁰.

Drosophila	MPFNAKSETDAKPEAPAPASEPAELESVDQKLEETHSKFREVDRQEQVLAEKAAEA	60
Human	MVSESH	6
Loligo	QEADELITAVDDTAGSAMETVTAKPKNIGDKIMDEALHELEKLPFWAILLCA	54
Drosophila	ASQRIA.VESTTTS.TTEAQE.TTTA.PVKKIEHVGEVVT.VIAERTG.T.GVVA.II	120
Human	HEALAAPVTTVA.VLPNS.TEPASPGEGKEDAFSL.EKFMNELHKIP.P.L.A.AI	66
Loligo	GVLFLVCGTYCCNR-ICRRRGKKDGK-KGLKGAVDLRGVLLGNSIKEK--PDLEELP	110
Drosophila	L.F.VVEGLIFF.VR.-FLKK.RT....G-.KG.DMKS....SAY....M...T	174
Human	VAV.LVLTCCE.I.J.KCLFKKKN..K..E..G.N.INMKD.KD..KTM.DQALK.-DDAE	125
Loligo	MNEDNEDAEKTSKSEVKLGKLYQSDYDFQKGLTIVNVIQAADLPGLMDSGTSDPVKYV	170
Drosophila	E.A.EGDE-.DKQ..Q...R.NFKLE..NSNS.A.T...EE..AL..G...IC.V...	233
Human	TGLT.G.EK.EP.E.E.....L.....NNQ.L.GI...E..AL..G...IC.V...	185
Loligo	Pep15	
Drosophila	LMPDKKKFETKVRKRTLNPFVNESFTFNKVPYADITGKTLVFAIYDFDRFSKHDQIGQV	230
Human	.L.....S.....T.....SL.....AMN.....IF.....E.....	293
Loligo	.L.....S.....Q.....SELGG.....M.V.....I.....EF	244
Loligo	C2A	
Drosophila	QVAMNSIDLGSVMEWRDLTSPDDAEKENKGLDGCIFSLRYVPTAGKLTVVILEAKNLK	290
Human	K.P.LCT...AQTI...V.VEGEGG...-.....S.....M...IC.V...	352
Loligo	K.P..TV.F.H.T...Q.AEKE--EQE.....C2B	302
Drosophila	MDVGGSLDPYVKISLMLNGKRIKKKTTVKKCTLNPNYNSFAFEVFPFQIQKVSIVTVT	350
Human	AI.Q.....L.....S.....S.....M...IC.V...	412
Loligo	H..Q.....L.....I..N.....S.....S.....QVV...	362
Drosophila	C2B	
Human	VDYDRHWTSEP IGRFTLGCNSTGTGLRHWSMDLANPRPRAQWHTLQEVPEKN	403
Loligo	IG.....CI.....MG.....S.....S.....KDPE.TDEILNMK	472
Human	L...KIGKND...KV.V.Y...AE.....VEE.VDAMLAVKK	422

quantities were injected. An 11-amino-acid peptide modelled after another part of the C2A domain (Pep11; see Fig. 1) was ineffective in blocking neurotransmission (Table 1).

Microinjection of various control substances provided further evidence for the specificity of Pep20 and Pep15 action. First, injection of the carrier solution did not inhibit release (Table 1). Second, a 23-amino-acid peptide that mimicked a positively charged region of the extracellular domain of the α_1 -subunit of the inhibitory glycine receptor¹⁴ failed to produce a reduction of the postsynaptic response (Table 1). Third, injections of a 20-amino-acid peptide which had the same amino-acid composition as Pep20 but a rearranged ('scrambled') peptide sequence, were also ineffective except in two experiments, in which there was an irreversible reduction of release (Fig. 2c, and Table 1). Pep20 and Pep15 are characterized by a conserved motif (xKKKKxxxK) with many basic residues reminiscent of the positively charged regions of other synaptic vesicle proteins¹⁵. But as a peptide of comparable basic charge density, namely the C-terminal tail of the α -latrotoxin receptor¹⁶ has no effect on release (Table 1), then nonspecific inhibition of release by a charge cluster is highly unlikely. We conclude that the inhibitory effects of Pep20 and Pep15 are due to their amino-acid sequence.

We next investigated the mechanism of action of Pep20. Direct recordings of presynaptic resting and action potentials did not reveal any significant effect on either following peptide injection. Although the sequences of Pep15 and Pep20 share little homology with the C2 domains of protein kinase C (PKC)¹¹, any inhibition of the enzyme by C2-domain peptides¹⁷ could affect synaptic transmission^{18,19}. Even at 2 mM, the peptide had no effect on PKC activity in crude synaptosomal preparations from squid optic lobes (data not shown). Further, bath application of a specific inhibitor of PKC, staurosporine (5 μ M), does not alter p.s.p. amplitude (M. Hans, D. Swandulla and G.J.A., unpublished results). Visualization of the spread of Texas-red-labelled Pep20 by confocal microscopy revealed that diffusion of the peptide within the terminal required several minutes (Fig. 3a). This diffusion-limited movement of peptide determines the speed of block, because the spread of Pep20 fluorescence and the degree of inhibition of release are highly correlated (Fig. 3b). This result suggests that inhibition of release is very rapid, on a timescale of seconds, once the peptide had reached active zones.

Fura-2 imaging²⁰⁻²² of resting and evoked Ca^{2+} levels in presynaptic terminals injected with Pep20 show that peptide injection has no effect on the time course or amplitude (mean ratio of Ca^{2+} rises after/before Pep20 injection of $0.97 \pm$

TABLE 1 Effects of microinjected peptides on synaptic transmission

Peptide	Sequence	Inhibition of transmission/ <i>n</i> *
Pep20	IKKKKTIVKKCTLNPPYNNES	12/12
Pep15	DKKKKFETKVHRKTL	3/4
Pep11	DMSGTSDPYVKVY	0/4
Scrambled		
Pep20	IEKYTKVCKTPNKLKNYKS	2/7
Glycine receptor α_1 -subunit peptide	LKEEKDLRYCTKHYNTGKFTCI	0/1
α -Latrotoxin receptor C-terminal peptide	CAKSANKNKKNDKEYVY	0/1
Carrier solution	—	0/2

Peptides were synthesized by Multiple Peptide Systems (San Diego, CA), or Neosystems (Strasbourg, France), and represented amino-acid positions 312–331 (Pep20), 174–188 (Pep15) and 158–170 (Pep11) of squid synaptotagmin (Fig. 1), amino acids 188–210 of the rat glycine receptor α_1 -subunit¹⁴, and residues 1,491–1,507 (with an additional N-terminal cysteine) of the rat α -latrotoxin receptor (neurexin)¹⁶. All peptides were dissolved in carrier solution containing FITC-dextran (see legend to Fig. 2) at a concentration of 20 mM, and injected repeatedly under fluorescence optics. Inhibition by Pep20 and Pep15 injections could be reversed after injection (Fig. 2a), but no recovery was seen in the two experiments in which scrambled Pep20 decreased neurotransmission.

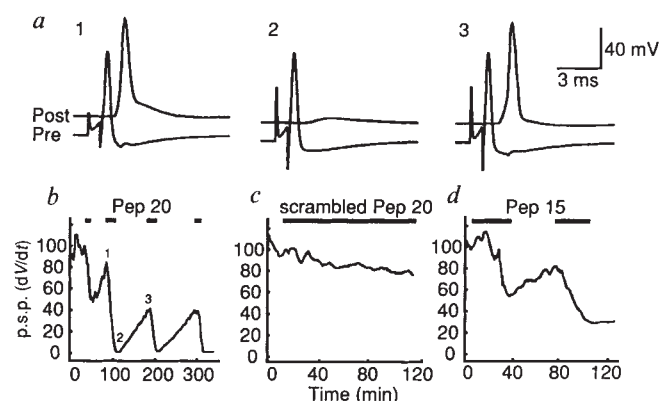
* An injection was judged to inhibit transmission if the rate of rise of the p.s.p. decreased by >50% after injection.

0.3 s.e.m.; $n = 3$) of internal calcium concentration ($[\text{Ca}^{2+}]_i$) signals evoked by presynaptic action potentials (Fig. 3c). Further, there is no significant change in resting Ca^{2+} levels (88 ± 14 nM before injection; 90 ± 13 nM after injection) or in the spatial distribution of $[\text{Ca}^{2+}]_i$ rises. Therefore Pep20 does not alter Ca^{2+} influx through voltage-gated Ca^{2+} channels or Ca^{2+} buffering within the terminal, but inhibits release at a step downstream from the increase in Ca^{2+} concentration.

Transmitter release constitutes one step in a cycle of exocytosis, endocytosis and vesicle transport^{23,24}. We used electron microscopy to determine where Pep20 affects this vesicle cycle. The typical morphology of active zones²⁵ was not obviously altered by microinjection of Pep20 (Fig. 3d) compared with the ineffective Pep11 (Fig. 3e). Analysis of the spatial distribution of synaptic vesicles at active zones by quantitative mor-

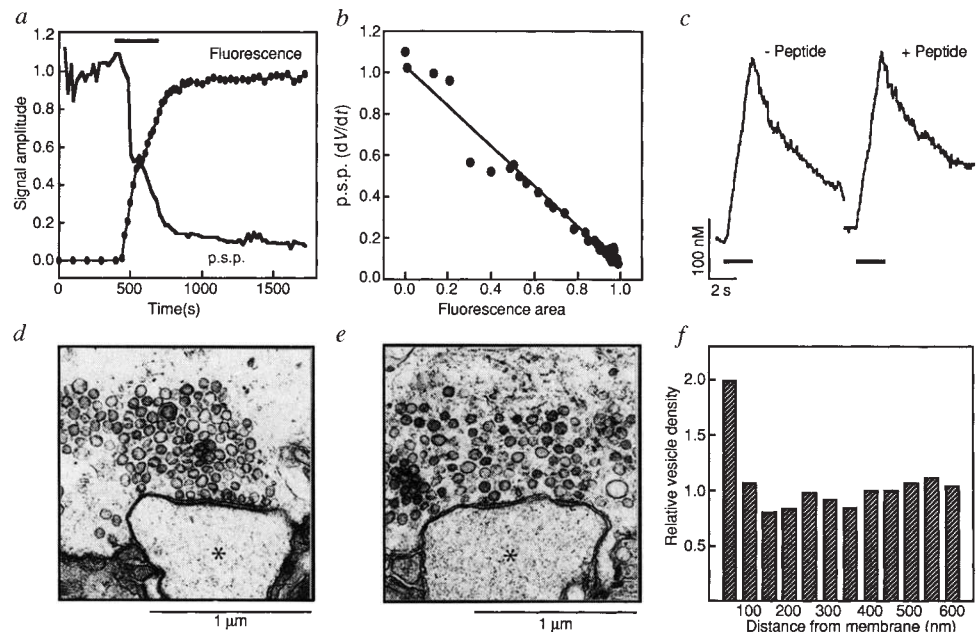
FIG. 2 Synaptotagmin peptides inhibit neurotransmitter release. **a**, Recordings of presynaptic (Pre) and postsynaptic (Post) electrical signals during microinjection of Pep20. Before injection, presynaptic action potentials elicited p.s.ps so large that they evoked postsynaptic action potentials (left). Immediately after peptide injection, p.s.ps decreased until their amplitude was insufficient to evoke a postsynaptic action potential (centre), but this inhibition was reversible (right). **b**, Time course of inhibition produced by Pep20. Four injections of the peptide, during the times indicated by bars, each produced a dramatic and reversible decrease in p.s.p. slope (dV/dt ; normalized to mean pre-injection value). The corresponding traces at time points 1, 2 and 3 are shown in **a**. **c**, Prolonged injection of scrambled Pep20 (Table 1) had little effect. **d**, Time course of inhibition of transmission produced by microinjection of Pep15. Note that longer periods of injection were required to affect transmitter release, suggesting a reduced efficacy for this peptide.

METHODS. Transmission at giant synapses of squid (*Loligo pealei*) stellate ganglia was examined using electrophysiological techniques¹². Presynaptic action potentials were evoked by current injected into the presynaptic axon through one microelectrode, while two other microelectrodes were used to measure electrical signals in the pre- and postsynaptic neurons. Data were stored and analysed by 'Tomahawk', a program written by T. Goldthorpe. The presynaptic recording electrode was filled with a carrier solution (200 mM potassium isethionate, 100 mM taurine and 50 mM HEPES buffer, pH 7.2) containing 20 mM peptide. A fluorescent indicator dye, fluorescein



isothiocyanate (FITC-dextran, M_r 10K, concentration 100 μ M; Molecular Probes), was added to the injection solution and examined with a fluorescent microscope (Nikon Optiphot) to monitor the exclusively presynaptic localization of the injected material. Solutions were delivered by pressure injection (Picospritzer, General Valve Co., New Jersey) using brief pulses (50–200 ms, 0.1–0.5 Hz, 20–60 psi) applied during the times indicated by the bars in **b–d**.

FIG. 3 Effects of Pep20 on Ca^{2+} influx and nerve terminal morphology. **a**, Correlation between diffusional spread of peptide and inhibition of release. Time course of inhibition of p.s.p. slope (—) and spread of fluorescently labelled Pep20 through the presynaptic terminal (●) upon peptide injection (duration indicated by bar). **b**, Plot of the normalized slope of the p.s.p.s versus the degree of peptide spread, measured simultaneously at various time points shown in **a**. From the intensity of peptide fluorescence, Pep20 concentrations of the order of 10^{-4} to 10^{-3} M were estimated to block neurotransmission. **c**, Peptide injection does not affect presynaptic calcium signals. Fura-2 imaging^{20–22} was used to measure rises in $[\text{Ca}^{2+}]_i$ evoked by trains of presynaptic action potentials. Time course of $[\text{Ca}^{2+}]_i$ rises, evoked by 50 Hz, 2 s trains (bars), is similar before and after peptide injection. **d** and **e**, Electron micrographs of active zones in terminals injected with Pep20 (**d**) or Pep11 (**e**). **f**, The number of synaptic vesicles found at increasing distances from the membrane of presynaptic terminals injected with Pep20 or Pep11 was measured for bin intervals of 50 nm. The relative spatial distribution of active zone synaptic vesicles was determined for each bin²⁶ by dividing the mean number of vesicles measured in each bin from Pep20-injected active zones ($n=103$) by the number measured in Pep11-injected active zones ($n=105$). This ratio differed significantly only for vesicles within 50 nm of the presynaptic membrane. **METHODS**. Pep20 was conjugated to Texas red dye (Molecular Probes) according to the instructions of the manufacturer. As the fluorescent peptide was not very effective in inhibiting release, a mixture of labelled (100 μM)



phometry²⁶ showed a similar overall distribution after both Pep20 and Pep11 injections, except that the number of vesicles closely adjacent to the presynaptic membrane (within 50 nm) was doubled in Pep20-injected terminals (Fig. 3f). Moreover, active zones in terminals injected with Pep20 had 5.00 ± 0.17 (mean $\pm$ s.e.m.) vesicles touching the presynaptic plasma membrane, whereas those in terminals injected with Pep11 had only 3.14 ± 0.12 (mean $\pm$ s.e.m.) such vesicles ($P < 0.001$). Therefore, Pep20 has no effect on the docking of vesicles at the active zones, but causes a build-up of docked vesicles. This indicates that Pep20 inhibits a later step of exocytosis, such as the initiation of release or subsequent vesicle-plasma membrane fusion.

Our results are evidence that synaptotagmin is involved in release, a result consistent with the proposal that this protein mediates Ca^{2+} -regulated vesicle-plasma membrane fusion⁶. Apparently, synaptotagmin is essential for neurotransmitter release from small synaptic vesicles, as found in squid nerve terminals, but not from the large dense-core vesicles of PC12 cells²⁷. Moreover, our study indicates that the C2 domain(s) of synaptotagmin is crucial for neurotransmitter release. Our results are consistent with this domain(s) binding to an acceptor protein at active zones and a competitive inhibition of this interaction by Pep20 and Pep15. From the effects of these peptides, we predict that the basic regions of the C2 domains contain important determinants of this protein-protein interaction. Although the identity of the presynaptic synaptotagmin acceptor is not yet known, it may be one of the presynaptic plasma membrane proteins known to bind synaptotagmin, such as the syntaxins⁹, the α -latrotoxin receptor⁷, or voltage-gated Ca^{2+} channels⁸. Our ultrastructural data show that the critical synaptotagmin-acceptor interaction occurs at a step close to (or at) the site of the partial reaction responsible for exocytosis, namely downstream from the attachment of synaptic vesicles at the presynaptic plasma membrane. Understanding the nature of this interaction should reveal how calcium regulates exocytosis in neurons

and unlabelled (20 mM) Pep20 was injected into the presynaptic terminal. Time-lapse imaging of peptide fluorescence was done with a Noran Odyssey confocal microscope (Middleton, Wisconsin), and the relative presynaptic area covered by fluorescent peptide was measured using digital image analysis software¹², while simultaneously recording elicited p.s.p.s¹³. For electron microscopy, tissue was fixed and prepared as before²². Two terminals were injected with Pep20, and two others with Pep11 as controls. Sections of 70 nm were acquired at 50- or 75- μm intervals along the length of each presynaptic terminal and examined on a JEOL electron microscope. Photographic prints of active zone images were digitized and analysed (Image-1 software, Philadelphia) as described²⁶.

and perhaps in other secretory cells.

Note added in proof: Recent antibody injection experiments, published after submission of this manuscript, provide evidence that synaptotagmin may have also a role in exocytosis of dense core vesicle contents³¹. □

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Continuous *c-fos* expression precedes programmed cell death *in vivo*

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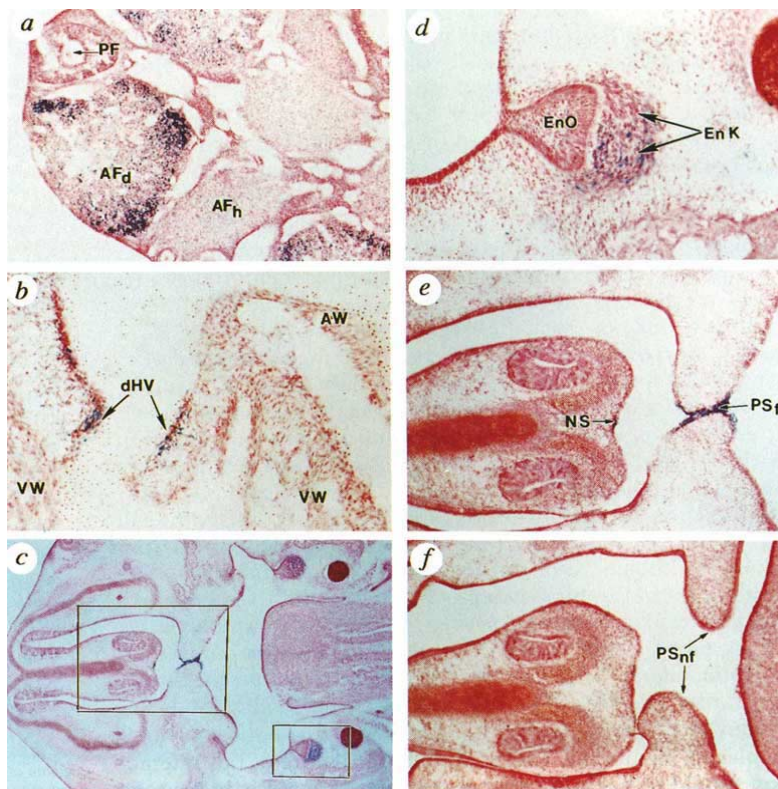
THE development of a multicellular organism involves a delicate balance among the processes of proliferation, differentiation and death. Naturally occurring cell death aids tissue remodelling, eliminates supernumerary cell populations and provides structural elements such as hair and skin. In the nervous system, selective cell death contributes to the formation and organization of the spinal cord and sympathetic ganglia¹, retina² and corpus callosum³. But cell death also occurs in several neuropathological conditions, such as amyotrophic lateral sclerosis⁴ and Alzheimer's disease⁵. Therefore an elucidation of the mechanisms responsible for cell death is critical for an appreciation of both normal development and neuropathological disorders. Using a *fos-lacZ* transgenic mouse⁶, we provide evidence showing that the continuous expression of Fos, beginning hours or days before the morphological demise of the cell, appears to be a hallmark of terminal differentiation

and a harbinger of death.

We previously reported that *fos-lacZ* was expressed, apparently continuously, in cell populations undergoing terminal differentiation culminating in death in skin, hair follicle and bone⁶. These and other results⁷⁻¹⁰ prompted us to examine other sites of naturally occurring cell death. In the ovary, Fos-lacZ-positive follicular cells are associated with atretic follicles/corpora lutea that are degenerating or regressing, whereas follicles containing an oocyte (PF) do not exhibit β -galactosidase activity (Fig. 1a). During embryonic development, several structures that contribute to organogenesis form transiently and are eliminated by programmed cell death and transdifferentiation. These include the heart valve cushion, the enamel knot which organizes tooth formation and the medial edge epithelium of the palate^{11,12}. *fos-lacZ* expression was detected in each of these structures at embryonic days 13 and 14 (Fig. 1b, e, respectively). The palate forms by midline fusion of two epithelial/mesenchymal shelves. At the point of fusion the medial edge epithelium disappears, permitting the underlying mesenchyme in the two individual shelves to fuse, thus forming a continuous structure^{12,13}. Because fusion proceeds in an anterior to posterior direction, the temporal relationship among *fos-lacZ* expression, palate fusion and cell death in the medial edge epithelium can be determined. *fos-lacZ* was not expressed in areas in which the two medial edge epithelia had not yet contacted (Fig. 1f). But in more anterior regions of the palate in which fusion had been initiated there was pronounced *fos-lacZ* expression in the medial edge epithelium (Fig. 1c, e). Expression was also observed in a small population of cells located at the midline of the nasal septum at the point of fusion of the nares (Fig. 1e).

Many agents, including plant and algal metabolites, pesticides and heavy metals, cause neurodegeneration. A subset of neurotoxins kill neurons by a hyperexcitatory action mediated by the glutamate receptor family¹⁴. To examine the relationship between excitotoxic cell death and *c-fos* expression, we deter-

FIG. 1 Naturally occurring sites of *fos-lacZ* expression during development. a, In the ovary, *fos-lacZ* expression was seen primarily in cells within an atretic follicle or corpus luteum that were degenerating (AF_d). In an atretic follicle/corpus luteum that is presumed to be in the hormonal stage (AF_h), little β -galactosidase activity was noted. No *fos-lacZ* expression was observed in an active primary follicle (PF, arrow). b, At E14, Fos-lacZ was detected in the developing heart valves (dHV). No *fos-lacZ* expression was noted in the surrounding ventricular (VW) or atrial (AW) walls. c, Low-power photomicrograph through the head of an E14 mouse showing the nasal and oral cavities. Boxed areas are shown in enlargement in d and e. d, At E14, *fos-lacZ* expression was seen in the enamel knot (EnK) of the developing tooth. No expression was observed in the overlying enamel organ (EnO). e, At the point of fusion of the palatal shelves (PS_f), expression of *fos-lacZ* was seen in cells of the medial edge epithelium. In addition, at the point of fusion of the nasal septum (NS), a small cluster of Fos-lacZ-positive cells were noted (arrow). f, No *fos-lacZ* expression was seen in the non-fused palatal shelves (PS_{nf}). This section is located 100 μ m from the section in e. Animal tissues were fixed and stained as described in ref. 26.



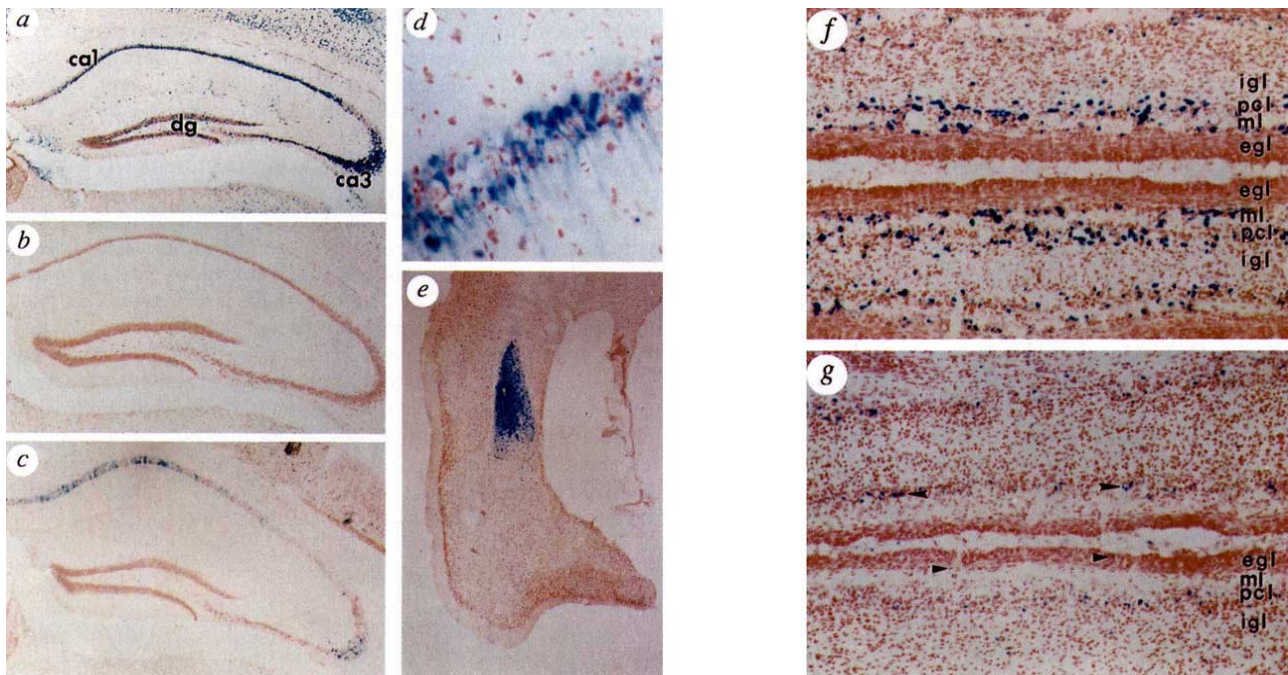


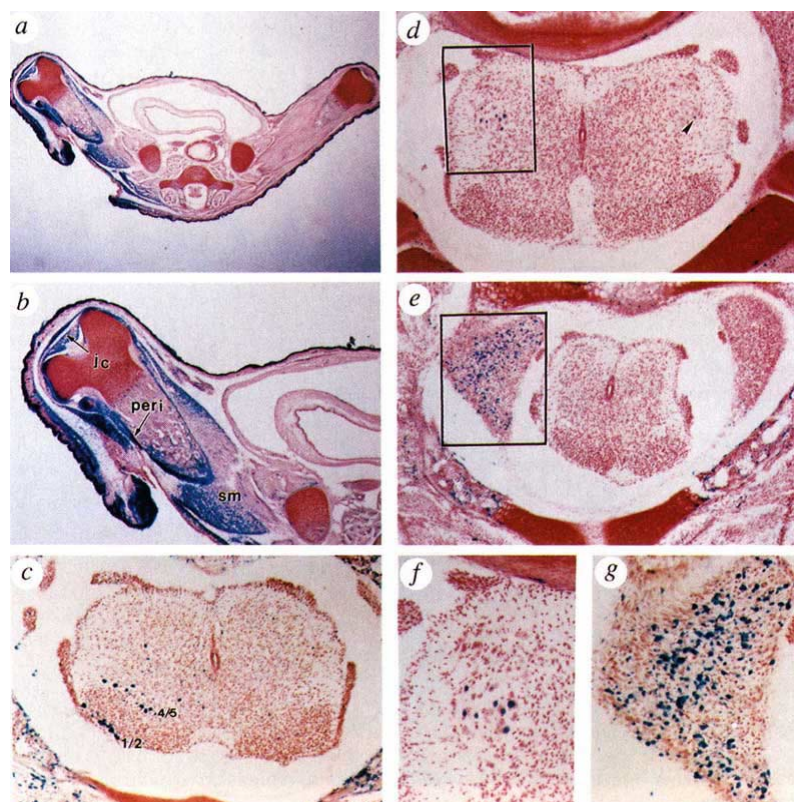
FIG. 2 Excitotoxic and genetic lesion of the CNS. *a*, Ninety minutes after systemic administration of kainic acid (KAI), most of the pyramidal neurons in ca1 and ca3 expressed *fos-lacZ*. About 50% of the granule cells of the dentate gyrus (dg) also expressed the transgene. *b*, Twenty-four hours after systemic KAI, *fos-lacZ* expression was absent from the dentate granule cells as well as from the pyramidal neurons of Ammon's horn. In the pyramidal cell layer of the hippocampus from 4–10 days after systemic administration of KAI there was a reinduction of *fos-lacZ*. *c*, 7 days after KAI injection, Fos-lacZ was seen in the ca1 and ca3 region of the hippocampus. The distribution of β -galactosidase in the pyramidal cells was cytoplasmic rather than nuclear. This is shown at a higher magnification in *d*. The shape of the pyramidal cells can be seen because the β -galactosidase product fills the entire cell body as well as their processes. *e*, Seven days after KAI, cytoplasmic *fos-lacZ* expression was noted in the dorsomedial

nucleus of the amygdala. *f, g*, Expression of *fos-lacZ* in the weaver mutant mouse. *f*, In this section through the anterior cerebellar lobules of a P4 heterozygous weaver mouse, *fos-lacZ* expression was detected in the mantle zone of the external granular layer (egl), the molecular layer (ml), the Purkinje cell layer (pcl) and the superficial internal granular layer (igl). *g*, In a similar region of the normal mouse, few Fos-lacZ-positive cells were observed (arrowheads).

METHODS. Adult Fos-lacZ mice were given intraperitoneal injections of kainic acid at epileptogenic doses (17 mg kg^{-1}). At 90 min, 5 h, 24 h, 2, 4, 7 and 10 days, animals were transcardially perfused, tissues processed, and histochemistry was done. At P4, normal mice (derived from $+/+ \times +/+$ matings) and heterozygous weaver mice (derived from $+/+ \times wv/wv$ matings) were transcardially perfused with 2% paraformaldehyde in 0.1 M PIPES buffer.

FIG. 3 Expression of *fos-lacZ* following sciatic nerve section. *a*, Low-power photomicrograph through lesion site. *fos-lacZ* was only induced ipsilateral to the lesion (left). *b*, High-power photomicrograph of the lesion site. *c*, Eight hours after surgery, expression of the *fos-lacZ* transgene was seen in laminae 1/2 and 4/5 of the dorsal horn of the spinal cord. *d*, 24 h after the sciatic nerve lesion, Fos-lacZ was seen in the degenerating motor neurons of the lumbar spinal cord, ipsilateral to the sciatic nerve lesion. Contralaterally, no increase was seen in the frequency of cells expressing *fos-lacZ*, which was usually 0–1 cells per section (arrowhead). A higher-power photomicrograph of the box is shown in *f*. Fos-lacZ was seen in the nucleus of about 50% of the motor neurons. Expression of the transgene was seen in the ipsilateral, but not the contralateral, dorsal root ganglion (*e*). A higher-power photomicrograph of the DRG neurons is shown in *g*.

METHODS. At P0, Fos-lacZ mice were anaesthetized using a combination of Avertin and hypothermia. When deeply anaesthetized, the sciatic nerve on the animal's left side was cut using a pair of fine microvanna scissors. The wound was closed with cyanoacrylate, and the animal was placed on a warming tray until mobile. At this time the animal was placed back in its home cage. Eight hours after surgery, half of the operated animals were killed and processed as in Fig. 1. After 24 h, the other half of the operated animals were killed and processed similarly.

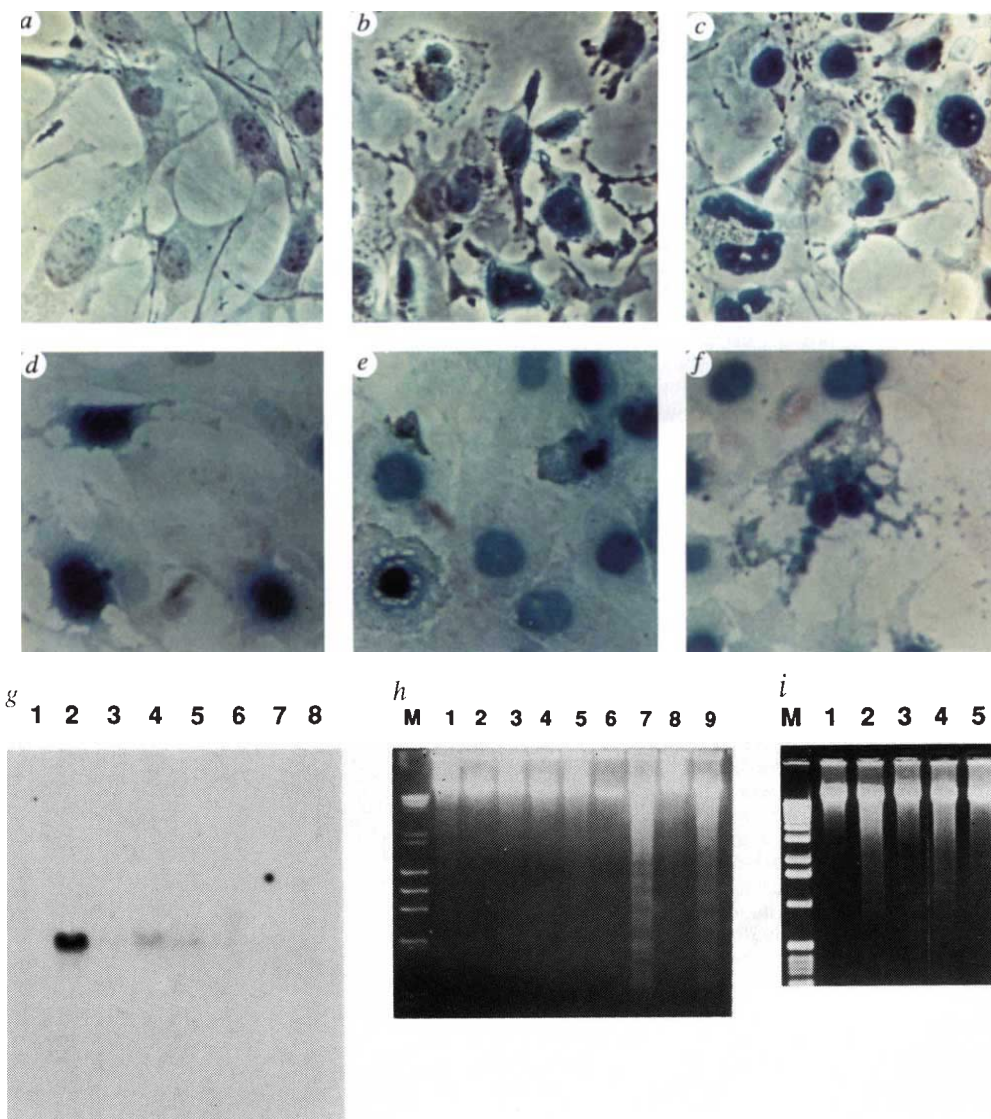


mined the temporal and spatial pattern of Fos-lacZ after administration of kainic acid. The brains of transgenic animals were examined at 1.5 h, 5 h, 24 h, 48 h, 7 d and 10 d after intraperitoneal injection of kainic acid. Ninety minutes after treatment, β -galactosidase activity was detected in several regions of the brain, including the hippocampus (Fig. 2a). At this time, Fos-lacZ was exclusively confined to neuronal nuclei. After 5 h, expression declined and Fos-lacZ was not detectable at 24 h (Fig. 2b). Throughout this period, the cytoarchitecture of the hippocampus remained intact. At 4 d post-treatment, and peaking at around 7 d post-treatment, *fos-lacZ* expression reappeared in specific regions of the limbic system: CA1, CA3 and to a lesser extent CA4 of the hippocampus as well as the dorsomedial nucleus of the amygdala (Fig. 2c-e). This pattern of *fos-lacZ* expression is coincident with the map of neurotoxicity described for kainic acid¹⁵. In contrast to the earlier expression, β -galactosidase activity was distributed throughout the entire cell (Fig. 2d). At this time, marked disorganization, pyknosis and neuronal loss was noted in the CA1 and CA3 pyramidal cell layers of the hippocampus. By 10 days, only a

few, sporadic, β -galactosidase-positive cells were observed in the hippocampus and amygdala (data not shown). These data suggest that a reinduction of *fos-lacZ* occurred before the onset of cell death in the neurons of the limbic system that are susceptible to kainic acid-mediated excitotoxicity.

Several genetic mutations in mice that result in cell death in the central nervous system have been described¹⁶. The *weaver* mouse is characterized by the death of virtually all midline cerebellar granule cells¹⁷. To investigate the association between genetically determined neurodegeneration and *c-fos* expression, Fos-lacZ mice were mated with homozygous female *weaver* mice. In the heterozygous *weaver*, there is extensive cell death although the process is more protracted than in homozygotes¹⁷. At P4, in the midline cerebellum of the heterozygous *weaver*-Fos-lacZ mouse, cells expressing β -galactosidase activity were detected in granule cells located in the mantle region of the external granular layer, the Purkinje cell layer, the molecular layer and the superficial regions of the internal granule layer (Fig. 2f). These are precisely the locations in which granule neurons degenerate in *weaver*¹⁷. In contrast, only a few Fos-lacZ-positive

FIG. 4 Association between cell death and *fos* expression *in vitro*. Fibroblasts from *fos-lacZ* mice were exposed to etoposide or phorbol ester and examined for β -galactosidase expression. *a*, 0.2% Dimethylsulphoxide (DMSO) for 6 h; *b*, 100 $\mu\text{g ml}^{-1}$ etoposide (Sigma) for 6 h; *c*, 100 nM phorbol ester for 6 h; *d*, etoposide for 6 h; *e*, *f*, etoposide for 12 h. In *e*, an extruded condensed nucleus is observed; in *f*, β -galactosidase can be seen in the cytoplasm of a disintegrating fibroblast. *g*, *h*, Relationship between *c-fos* expression and nucleosome fragmentation *in vitro*. PC12 cells were treated with 100 $\mu\text{g ml}^{-1}$ etoposide for the indicated times before isolation of RNA and DNA. The levels of *c-fos* mRNA were determined by northern analysis (*g*); lanes 1, 0.2% DMSO for 1 h; 2, 100 ng ml^{-1} nerve growth factor (Collaborative Research) for 1 h; 3, etoposide for 0.5 h; 4, etoposide for 1 h; 5, etoposide for 2 h; 6, etoposide for 4 h; 7, etoposide for 6 h; 8, etoposide for 8 h. *h*, DNA was extracted from etoposide-treated cells and analysed by gel electrophoresis and ethidium bromide staining. Lanes 1, etoposide for 1 h; 2, 0.2% DMSO for 2 h; 3, etoposide for 2 h; 4, etoposide for 4 h; 5, etoposide for 6 h; 6, etoposide for 8 h; 7, etoposide for 12 h; 8, 0.2% DMSO for 24 h; 9, etoposide for 24 h; M, DNA markers (New England Bio-Labs). *i*, Nucleosome laddering in fibroblasts cultured in 0.1% fetal bovine serum for 48 h. Lanes 1, normal rat fibroblasts; 2, rat fibroblasts transformed by CMVc-*fos*; 3, rat kidney cells transformed by *v-ras*^{Ki}; 4, rat fibroblasts, containing a *c-fos* conditional expression vector (L1-3c-*fos*) based on a *lac* activator protein (details of this cell line will be published separately) cultured in the absence of IPTG, under which condition *c-fos* is expressed; 5, rat fibroblasts containing the expression vector cultured in the presence of IPTG which results in repression of *fos* expression.



METHODS. Fibroblasts from 14-day *fos-lacZ* embryos under a standard 3T3 regime. After crisis, cells were cloned twice to establish the *fos-lacZ* line. Cells were fixed and stained for β -galactosidase as described previously⁶. DNA extraction and agarose gel analysis were done as described^{27,28}.

cells were detected in the cerebellum of normal littermates containing the *fos-lacZ* transgene (Fig. 2g). This is consistent with the relatively small amount of naturally occurring cell death that occurs in cerebellar granule cells.

Neuronal death occurs naturally in the spinal cord and dorsal root ganglia (DRG) during development through a process that involves target-derived trophic factors¹⁸. A few Fos-lacZ-positive cells were detected in these locations in transgenic mice. We induced a synchronous wave of cell death in the spinal cord and DRG by lesion of the sciatic nerve. On the day of birth, transgenic animals were given unilateral scissor-cut lesions of the sciatic nerve and examined at 8 and 24 h post-surgery. After 8 h, extensive *fos-lacZ* expression was observed throughout the ipsilateral limb and body, including skeletal muscle, bone, joint capsule and periosteum (Fig. 3a, b). In the CNS, 8 h after sciatic nerve lesion, *fos-lacZ* expression was detected in laminae 1/2 and 4/5 of the dorsal horn of the spinal cord (Fig. 3c). This induction was specific both with regard to laterality and level in the spinal cord. *fos-lacZ* expression was only observed in the ipsilateral spinal cord between L3 and S2, consistent with the denervation of the sensory zones supplied by the sciatic nerve. Laminae 1/2 at this level contain neurons that receive afferent innervation primarily from cutaneous nociceptive zones, whereas neurons in laminae 4/5 respond to many sensory stimuli and form the proper sensory nucleus¹⁹. At 24 h post-surgery, the extensive *fos-lacZ* expression in the ipsilateral skeletal muscle, bone cells, and dorsal horn of the spinal cord largely disappeared. In the spinal cord ipsilateral to the lesion, *fos-lacZ* expression was detected in about half the somatic alpha-motor neurons of lamina 9 in a level-specific manner (Fig. 3d, e). On the side contralateral to the lesion, only a few (0–2 per section) motor neurons expressed *fos-lacZ*. Fos-lacZ was also observed in the ipsilateral DRG from L2–L4 (Fig. 3f, g). Cells of the DRG die after sciatic nerve lesion because of the absence of a target-derived trophic influence. Indeed, nerve growth factor (NGF) rescues DRG neurons after sciatic nerve lesion²⁰.

To determine whether *fos-lacZ* expression preceded cell death, cultured cells were treated with the drug etoposide, which elicits apoptosis in a number of cell types²¹. *fos-LacZ* expression was first detected 2 h after addition of etoposide, a delay of 1 hour compared to the induction observed with serum or phorbol ester. Subsequently, β -galactosidase activity (Fig. 4a–c) as well as *c-fos* and *fos-lacZ* messenger RNAs remained elevated throughout the time course examined (data not shown). By 12 h, most of the cells expressed *fos-LacZ* and there was an obvious reduction in cell number. At this time, β -galactosidase activity could be detected in condensed nuclei (Fig. 4e) and cytoplasmic remnants (Fig. 4f). In PC12 cells treated with etoposide (Fig. 4g, h), the levels of *c-fos* mRNA increased 1 h after treatment and remained above basal levels for at least 6 h. Nucleosome laddering, which has been widely used as an index of apoptotic cell death²², was not evident until after 8 h of treatment. Thus, *c-fos* was expressed at least 6 h before the first biochemical evidence of cell death.

The *myc* oncogene may induce apoptosis in cultured fibroblasts²². To establish a functional link between *c-fos* and cell death, we analysed the effect of serum-deprivation on cells transformed by *c-fos* (Fig. 4i). Low levels (7.2%) of cell death were observed in normal rat fibroblasts deprived of serum for 2 days. In contrast, 79% of cells transformed by a vector expressing *c-fos* died under the same conditions. Nucleosome laddering was clearly detected in extracts made from the *fos*-transformed cell cultures but not in extracts made from normal fibroblasts. DNA fragmentation was specifically associated with *fos* expression by two criteria. First, little or no laddering was detected in serum-deprived *ras*-transformed fibroblasts (Fig. 4i). Second, using fibroblasts containing a conditional vector that expressed *fos* under the control of isopropyl- β -D-thiogalactoside (IPTG), nucleosome laddering was only evident when Fos was present (Fig. 4i).

Expression of the *fos* proto-oncogene transcription factor has been associated with a variety of biological responses, including proliferation, differentiation and neural excitation²³. In these situations, *fos* expression is transient. Here we show that β -galactosidase activity in a *fos-lacZ* mouse also marks populations of moribund and dead cells both in natural and in experimentally induced situations. In this case, expression of the gene appears to be continuous and precedes other morphological and biochemical markers of cell death by many hours or days. Thus, the transgenic mouse provides a convenient resource for investigation of cell death with single cell resolution. Here we have applied this technology to the analysis of cell death during development, following excitotoxic or surgical lesion, as well as in a neurodegenerative mutant. The finding that *fos-lacZ* was expressed in hypertrophic chondrocytes undergoing programmed cell death during bone formation and epiphyseal bone growth, indicated that *c-fos* may play a role in the elimination of these cells. Indeed, recent reports show that the major defect in *c-fos* null mice is an apparent osteopetrosis, one feature of which is persistence of chondrocytes within the bone matrix and epiphysis^{24,25}. This phenotype may arise from a reduction in programmed cell death of hypertrophic chondrocytes. Similarly, aberrant tooth formation and defective folliculogenesis in the *fos*-null mice may be related to the expression of *c-fos* reported here. A major question raised by these observations concerns the functional role of Fos in the process of cell death. Three possible scenarios can be envisaged. First, *c-fos* expression may have no causal relationship to cell death, but it may merely be a reflection of a breakdown of intracellular signal transduction. Second, the signalling pathway that leads to cell death may result in a coincidental induction of *c-fos* and perhaps other immediate-early genes. Third, the *c-fos* product may be a required component of the gene regulation pathway that leads to cell death in some circumstances. As there is considerable information available concerning the intracellular signalling pathways that regulate *c-fos* expression, it may be possible to use the transgene as a molecular indicator to dissect the signal transduction processes involved in the earliest stages of programmed cell death. □

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p70^{S6k} function is essential for G1 progression

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AN essential step in the pathway by which growth factors trigger cellular proliferation is the induction of high levels of protein synthesis¹⁻³. This appears in part to be controlled by multiple phosphorylation of the ribosomal protein S6 (refs 4, 5). The main kinase responsible, p70^{S6k} (refs 6-8), is activated through the phosphorylation of four sites clustered in a putative autoinhibitory domain⁹, which is mediated by a signalling pathway distinct from those used by other well characterized mitogen-activated serine/threonine kinases (such as p42/p44^{mapk} or p90^{rsk}; refs 10, 11). Here we investigate the role of p70^{S6k} in the mitogenic response. Microinjection of quiescent rat embryo fibroblasts with any of three distinct polyclonal antibodies to p70^{S6k} abolishes serum-induced entry into S phase of the cell cycle. This effect is preceded by almost complete abrogation of the activation of protein synthesis and the expression of an essential immediate early gene product, *c-fos*. The inhibitory effect on DNA synthesis is also elicited by microinjection of the antibodies late in G1 phase, consistent with the finding that p70^{S6k} activity remains high throughout G1.

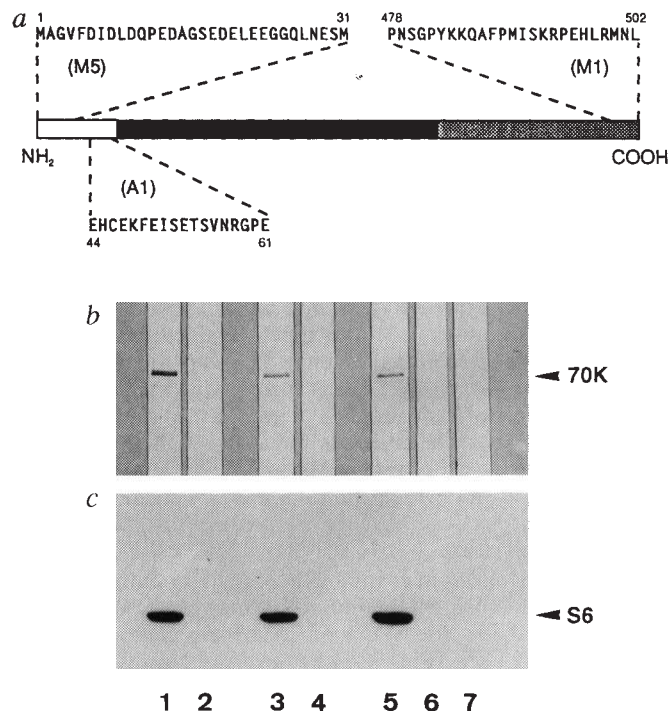
Antibodies that impede a protein function can be used to assess its importance *in vivo*¹²⁻¹⁴. We raised monospecific anti-

bodies against three peptide sequences derived from the C terminus (M1), the N terminus (M5) and a core sequence (A1) of rat p70^{S6k} (Fig. 1a). Purified IgGs from all three antibodies specifically recognized recombinant rat p70^{S6k} on western blots (Fig. 1b; lanes 1, 3 and 5) and immunoprecipitated S6 kinase activity (Fig. 1c; lanes 1, 3 and 5). These reactions were blocked by competing peptide (Fig. 1b and c; lanes 2, 4 and 6) or, in the case of the A1 antibody, after antigen-specific IgGs were removed by peptide affinity purification (A1⁽⁻⁾ IgGs; Fig. 1b and c, lane 7). Although all three antibodies immunoprecipitated p70^{S6k}, in each case total S6 kinase activity was inhibited ≥99% in the immune complex assay (see Fig. 1 legend).

As all the p70^{S6k} antibodies recognized the native enzyme, the effect of each antibody on serum-induced mitogenesis was investigated by microinjecting it with an inert marker antibody into the cytoplasm of quiescent REF-52 cells. Cells injected with any of the three antibodies failed to enter S phase as monitored by the incorporation of 5-bromo-2-deoxyuridine (BrdU) into DNA. Surrounding cells that had not been injected display normal BrdU nuclear staining (Fig. 2; compare a, c, e with b, d, f). This effect is specific for p70^{S6k}, because cells injected with negative A1⁽⁻⁾ IgGs (Fig. 2; compare g and h) or the marker antibody alone (data not shown) initiated S phase normally. In addition, all three antibodies specifically recognized p70^{S6k} on western blots of total cell extracts from REF-52 cells (data not shown). The most potent inhibitory antibodies were A1 (94%) and M5 (92%), followed by M1 (85%) (Table 1). In the A1⁽⁻⁾ injected cells 5% did not enter S phase; this amount is comparable to the normal BrdU incorporation profile of these cells¹⁴. We confirmed the specificity of antibody inhibition by co-injection of the A1 antibody with purified rat p70^{S6k} or the A1 peptide. In both cases inhibition by A1 antibody was reversed, allowing

FIG. 1 Anti-peptide p70^{S6k} polyclonal antibodies. a, Diagram of p70^{S6k} indicating positions of peptides used to raise polyclonal antibodies. The N terminal (white), catalytic (black) and regulatory (stippled) domains are shown. b, Western blot analysis and c, immune-complex S6 kinase assay using IgGs: M1 (lanes 1 and 2), M5 (lanes 3 and 4), A1 (lanes 5 and 6) and A1⁽⁻⁾ (lane 7) in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4 and 6) of competing peptide antigen.

METHODS. Synthetic peptides corresponding to amino acids 1-31 (M5) 44-61 (A1) and 478-502 (M1) of rat p70^{S6k} (ref. 22) were used to raise polyclonal antibodies in Chinchilla rabbits as described²³. IgGs were purified on protein A-Sepharose²⁴ (Pharmacia) and dialysed extensively against phosphate-buffered saline (PBS). The negative A1 IgG (A1⁽⁻⁾) was prepared by passing A1 IgGs three times over the same peptide affinity column, which comprised the antigen peptide coupled to cyanogen bromide-activated Sepharose (Pharmacia; 0.5 mg peptide per ml of gel), and collecting the flow-through. Under these conditions it was not possible to release the positive A1 IgGs from the column by standard protocols²⁴. Activated rat p70^{S6k} was expressed by infection of Sf9 cells with a baculovirus recombinant rat p70^{S6k} (S. C. Kozma *et al.*, manuscript submitted). Whole cell extracts were prepared 24 h after infection in buffer A (50 mM Tris-HCl (pH 8.0) at 4 °C), 120 mM NaCl, 1 mM EDTA, 5 mM EGTA, 20 mM NaF, 15 mM pyrophosphate, 30 mM p-nitrophenylphosphate, 1 mM benzamide, 0.1 mM phenylmethylsulphonylchloride and 1% Nonidet-P40 (S. C. Kozma *et al.*, manuscript submitted). For western blots, 1.3 µg total protein extract containing ~2.6 ng p70^{S6k} and a specific activity of 380 pmol min⁻¹ mg⁻¹ protein was electrophoresed on 15% acrylamide gels²⁵, transferred to Immobilon-P and blocked with non specific proteins²⁶. Blots were incubated with the appropriate IgG preparation (480 ng ml⁻¹) in PBS containing 0.5% Tween-20 (PBS-Tween) overnight at 4 °C, in the absence or presence of 5 µM competing peptide. The membrane was washed in PBS-Tween and treated with swine anti-rabbit immunoglobulins conjugated with alkaline phosphatase (DAKO), 1:1,000 in PBS-Tween, for 1 h at room temperature, washed and developed²⁷. For immunoprecipitation, aliquots of the baculovirus-infected Sf9 cell extracts containing 5.7 units of S6 kinase activity (one unit is equivalent to 1 pmol of P_i incorporated into S6 per min) were diluted to a final volume of 200 µl with buffer A containing 1% bovine serum albumin (BSA) and 21 µg of the appropriate IgG preincubated for 15 min on ice in the absence or presence of 5 µM competing peptide antigen. After 2 h incubation on ice, IgGs were precipitated with protein A-Sepharose²³ and washed twice with buffer A containing 1% BSA and twice with the same buffer containing 1 M NaCl. Pellets were then prepared for S6 kinase assay using rat liver 40S



ribosomal subunits²³; S6 phosphorylation was determined by electrophoresis and autoradiography²⁵. In a parallel experiment purified rat p70^{S6k} (ref. 25) was labelled by autophosphorylation with [³²P]ATP²³ and the amount of kinase and total activity precipitated by each antibody determined. Immunoprecipitation was carried out as described²³, using 3.2 units of kinase for each reaction. The M1, M5 and A1 antibodies immunoprecipitated 1.4, 26.6 and 6.8% of the total kinase present, respectively. Of the total p70^{S6k} activity in the M1, M5 and A1 immunocomplex only 0.33, 0.44 and 1.04%, respectively, were detected in the S6 kinase assay.

TABLE 1 Per cent inhibition of DNA synthesis, protein synthesis and *c-fos* expression in antibody-injected REF-52 cells

Inhibition of DNA synthesis			
Antibody	Cells inhibited/ cells injected		Inhibition (%)
M1	26/30, 48/50 19/28, 11/13		85
M5	25/28, 19/22 41/46, 54/55		92
A1	31/34, 30/30 26/29, 12/12		94
A1 ⁽⁻⁾	3/41, 2/56		5
A1 + rat p70 ^{s6k}	2/43, 3/37		6
A1 + A1 peptide	2/26, 2/36		7
Inhibition of protein synthesis			
Antibody	c.p.m. incorporated		Inhibition (%)
	Expt 1	Expt 2	
A1	1,795	1,363	87
A1 ⁽⁻⁾	10,835	10,251	7
Inhibition of <i>c-fos</i> expression			
Antibody	Cells inhibited/ cells injected		Inhibition (%)
A1	45/47, 29/31		95
A1 ⁽⁻⁾	3/39, 4/38		9

Antibodies were microinjected into the cytoplasm of serum-starved REF-52 cells and the cells stimulated with FCS as described in Fig. 2 legend. Cells were scored for either DNA synthesis (Fig. 2), protein synthesis (see later) or for the nuclear expression of *c-fos* protein by immunofluorescence²¹. The A1 antibody block was relieved with either rat p70^{s6k} (50 nmol) or A1 peptide (5 μ mol) by mixing the antigen with the antibody and immediately microinjecting the two components into serum-starved REF-52 cells followed by serum stimulation (Fig. 2 legend). Per cent inhibition was calculated from the results of independent experiments as indicated. To determine the amount of protein synthesis, 220–280 REF-52 cells grown on a 2 mm² glass chip were serum-starved (Fig. 2 legend). Every cell on each chip was microinjected with either A1 or A1⁽⁻⁾ antibody. The chip was then transferred to 5 μ l DMEM medium containing 250 μ Ci ³⁵S-methionine (Amersham). After 15 min, 5 μ l of the same medium containing 20% FCS or PBS was added to each chip so that the total volume in each case was 10 μ l. After 2 h serum stimulation, each chip was rinsed twice with 30 μ l PBS, and cells were extracted in 0.5% SDS, 1% Nonidet-P40, 1% β -mercaptoethanol, heated at 100 °C for 5 min, and proteins precipitated on ice with 5% trichloroacetic acid (TCA) in the presence of 0.5% bovine serum albumin carrier for 10 min. The pellet was resuspended in 5% TCA, heated at 100 °C for 5 min to hydrolyse [³⁵S]Met-tRNA, and the pellet resuspended; the amount of ³⁵S-methionine incorporated into protein was counted by liquid scintillation spectroscopy. For each experiment the amount of ³⁵S-labelled protein was normalized to 250 cells, and the per cent inhibition calculated after subtracting resting cell levels. For the two experiments shown, ³⁵S-incorporation in 250 non-injected, non-serum-stimulated resting cells was 3,009 and 2,888 c.p.m., respectively, and for 250 non-injected, serum-stimulated cells was 14,860 and 13,845 c.p.m., respectively.

the cells to enter S phase (Table 1). Injection of p70^{s6k} 24 h after injection of A1 antibody was 80–85% as efficient at rescuing S phase as co-injection of the kinase with the antibody (data not shown). A similar inhibitory effect was observed by microinjection of each of the four antibody preparations into either Swiss mouse 3T3 cells or a human cell line, HE10/23 (data not shown). In the rare cases where injection of specific p70^{s6k} antibodies did not block entry into S phase, the nuclei of these cells contained multiple elongated nucleoli, possibly reflecting a stress response associated with increased ribosome biogenesis. The inhibitory effect of these antibodies does not reflect a delay in the onset of DNA synthesis, because the block in BrdU incorporation was evident up to 60 h post-serum induction. By 72 h a number of cells had initiated DNA synthesis, concomitant with diminished marker antibody staining. This result is indica-

tive of antibody turnover¹⁵ and demonstrates that the inhibitory effects of the antibodies are reversible and non-toxic.

Our data indicate that abrogation of p70^{s6k} function blocks an essential step in mitogenesis. To test whether this block was exerted on translation, we investigated the effect of antibody injection on the activation of protein synthesis and the expression of an essential immediate early gene product, *c-fos*. A1 IgG suppressed activation of protein synthesis by almost 90% and blocked *c-fos* expression in 95% of cells injected (Table 1). By contrast, both responses were virtually unaffected by injection of the A1⁽⁻⁾ IgG (Table 1). However, as high rates of protein synthesis are also required for cells to transit G1 (ref. 2), and S6 remains fully phosphorylated during this time¹⁶, p70^{s6k} function would be expected to be essential throughout G1. We therefore measured p70^{s6k} activity and the effect of microinjection of the A1 antibody at selected times after serum stimulation (Fig. 3). The results show that the kinase remains maximally activated up to 14 h after serum induction, a time just before the first cells enter S phase (~16 h). The A1 antibody

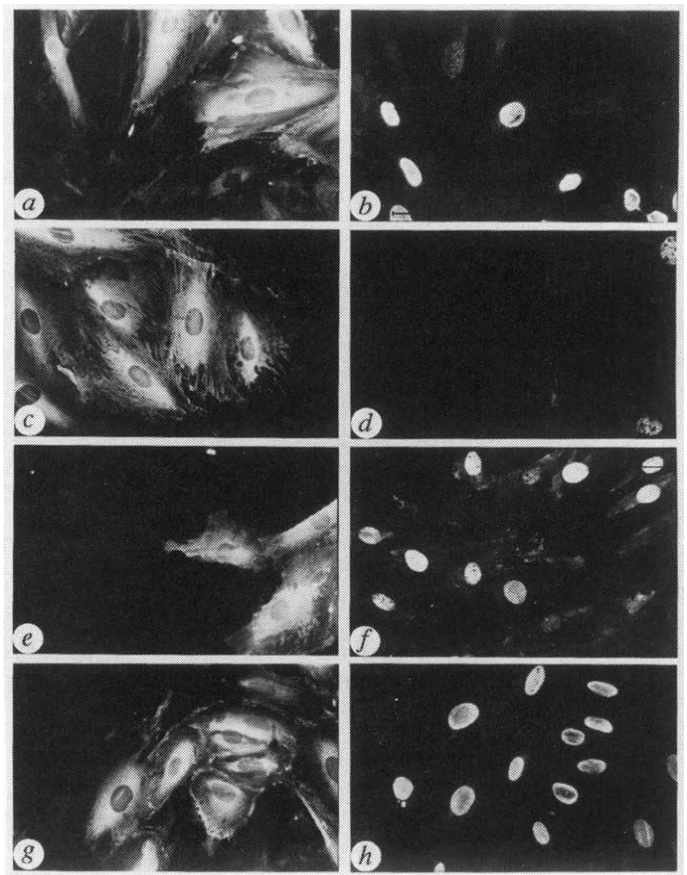


FIG. 2 Anti-p70^{s6k} immunoglobulins inhibit serum-induced initiation of DNA synthesis in synchronized REF-52 cells. REF-52 cells, deprived of serum for 36 h, were microinjected with the IgG fraction of antiserum M1 (a and b), M5 (panels c and d), A1 (panels e and f) or A1⁽⁻⁾ (g and h). After 1 h, cells were stimulated with fetal calf serum (FCS) and were fixed and stained 32 h later for incorporation of 5-BrdU and an inert antibody marker. Fluorescence micrographs are shown of injected cells stained for inert marker rabbit immunoglobulins (a, c, e and g) or BrdU (b, d, f and h).

METHODS. REF-52 cells were grown in DMEM as described¹⁴. After subculture onto glass coverslips, cells were synchronized by serum starvation for 36 h^{28,29}, and injected with the appropriate IgG fraction into the cytoplasm (prepared as for Fig. 1): M1, 2.0 mg ml⁻¹; M5, 1.0 mg ml⁻¹; A1, 1.0 mg ml⁻¹; A1⁽⁻⁾, 1.0 mg ml⁻¹. An inert rabbit marker antibody was included in the injection buffer to identify injected cells. Cells were then transferred to DMEM containing 10% FCS supplemented with BrdU fixed 32 h later and stained as described¹⁴.

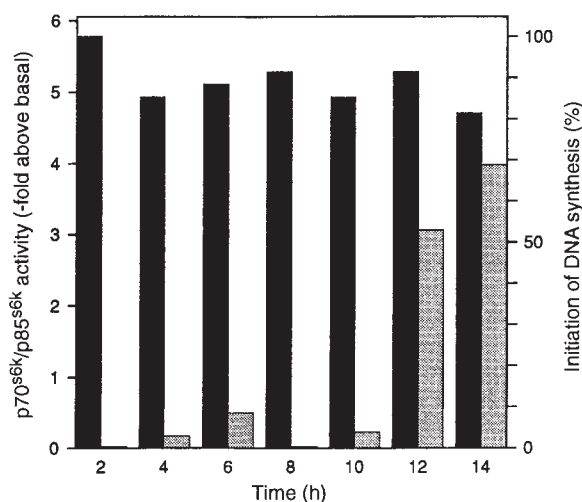


FIG. 3 p70^{s6k} activity remains high through G1 and injection of anti-p70^{s6k} immunoglobulins at any time during G1 inhibits serum-induced initiation of DNA synthesis. REF-52 cells were deprived of serum for 36 h and then induced to re-enter the cell cycle by FCS treatment. At selected time intervals, cells were either extracted and assayed for p70^{s6k} activity (black bars), or microinjected with A1 immunoglobulins and scored for the ability to initiate DNA synthesis (stippled bars).

METHODS. REF-52 cells were cultured, synchronized and stimulated with 10% FCS as described in Fig. 2 legend. For immunoprecipitation of p70^{s6k} activity, cells were extracted at selected times in buffer A²³. Cell extract protein 15 µg was immunoprecipitated with 5 µl M5 serum and the immunoprecipitates assayed against rat liver 40S ribosomal subunits²³. To assess the effect of antibody microinjection on G1 progression, cells were microinjected at selected times with A1 IgGs (1.0 mg ml⁻¹) and after continued incubation in 10% FCS, scored for their ability to initiate DNA synthesis as described for Fig. 2. Each value represents the mean of at least two experiments, in which the minimum number of cells injected in each experiment was 20. Microinjection of Al⁽⁻⁾ IgGs at the same concentration had no effect on S phase progression.

totally abrogated entry into S phase when it was injected during the first 10 h after serum induction (Fig. 3). This effect dropped to 50% and 30% by 12 and 14 h post-serum induction, respectively. However, injection of higher concentrations of antibody at these later times totally blocked entry into S phase (data not shown). This difference may reflect increased levels of kinase as cells progress towards S phase.

Together, our results indicate that p70^{s6k} function is essential throughout G1 and demonstrate the importance of this enzyme in the mitogenic response *in vivo*. The principal target of this enzyme is the 40S ribosomal protein S6 (refs 10, 17), therefore p70^{s6k} antibodies may exert their effect on protein synthesis through inhibition of S6 phosphorylation. A minor isoform of p70^{s6k} encoded by the same gene, termed p85^{s6k}, contains a nuclear targeting sequence, is activated by mitogenic stimulation⁷ and may participate in the regulation of nuclear S6 phosphorylation¹⁸. As these antibodies were raised against protein sequences common to both isoforms, they should crossreact with p85^{s6k}, as in the case of the M5 antibody⁷. The contribution of this molecule to the S6 phosphorylation response and its possible role in the nucleus, if any, need to be assessed.

The p70^{s6k} is under the control of a complex regulatory program^{19,20} essential for cell-cycle progression into S phase, member of a signalling pathway distinct from that of other well characterized mitogen-activated serine/threonine kinases. It remains to characterize the components involved in transduction from the receptor to the p70^{s6k} and to determine the point of bifurcation between these different pathways. □

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A novel dimer configuration revealed by the crystal structure at 2.4 Å resolution of human interleukin-5

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INTERLEUKIN-5 (IL-5) is a lineage-specific cytokine for eosinophilopoiesis and plays an important part in diseases associated with increased eosinophils, such as asthma^{1,2}. Human IL-5 is a disulphide-linked homodimer with 115 amino-acid residues in each chain². The crystal structure at 2.4 Å resolution reveals a novel two-domain structure, with each domain showing a striking similarity to the cytokine fold found in granulocyte macrophage³ and macrophage⁴ colony-stimulating factors, IL-2 (ref. 5), IL-4 (ref. 6), and human⁷ and porcine⁸ growth hormones. IL-5 is unique in that each domain requires the participation of two chains. The IL-5 structure consists of two left-handed bundles of four helices laid end to end and two short β-sheets on opposite sides of the molecule. Surprisingly, the C-terminal strand and helix of one chain complete a bundle of four helices and a β-sheet with the N-terminal three helices and one strand of the other chain. The structure of IL-5 provides a molecular basis for the design of antagonists and agonists that would delineate receptor recognition

determinants critical in signal transduction. This structure determination extends the family of the cytokine bundle of four helices and emphasizes its fundamental significance and versatility in recognizing its receptor.

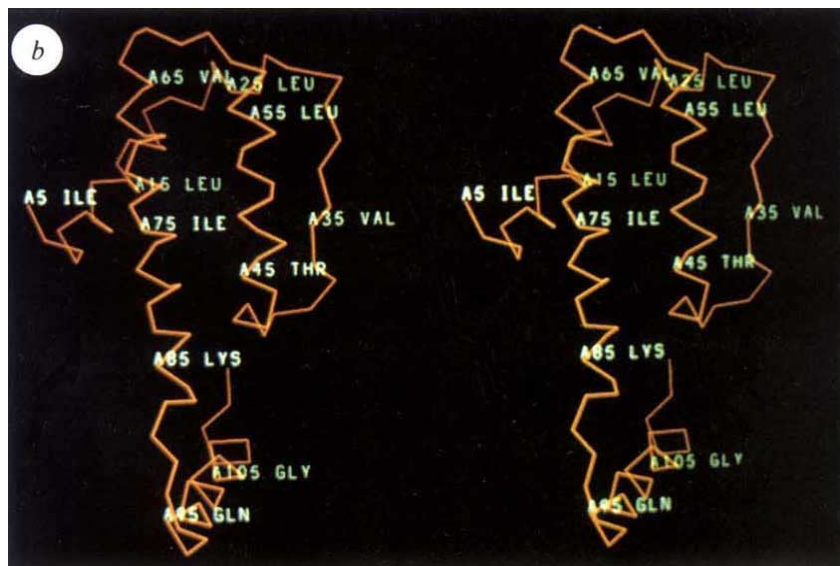
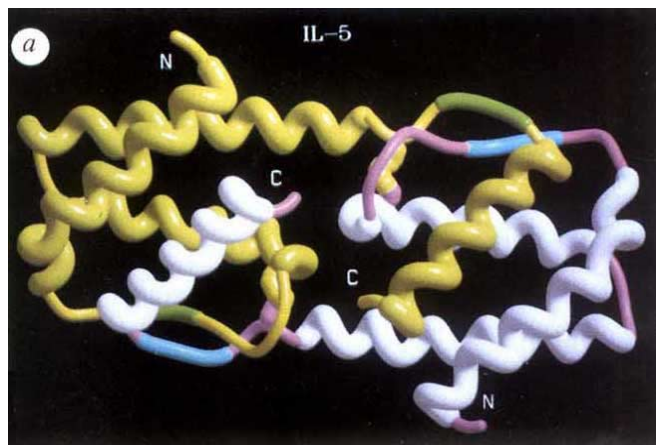
Human recombinant IL-5 was purified and crystallized as described earlier^{9,10}. Details of the structure determination are described in Table 1. The IL-5 dimer is an elongated ellipsoidal disk with approximate overall dimensions of $65 \times 30 \times 20 \text{ \AA}^3$. The only two cysteine residues in IL-5 form two intermolecular disulphide bonds which link the first cysteine of one chain to the last cysteine of the other^{11,12}. The crystal structure of IL-5 reveals an intimate dimer configuration in which each domain contains secondary structural elements from both chains (Fig. 1a). This intimate dimerization is reminiscent of two other structurally unrelated cytokines: IL-8 (ref. 13) and interferon- γ (ref. 14). The core of IL-5 is two left-handed bundles of four α -helices, laid end to end, and two short antiparallel strands packed on opposite sides of the dimer. Beginning at the N terminus, one polypeptide chain of IL-5 folds to form three α -helices (helices A, B and C) and one β -strand ($\beta 1$), all tightly packed together (Fig. 1b). Residues from Cys 86 to the C terminus form one strand ($\beta 2'$) and one helix (helix D') that pack against the other monomer chain to complete a bundle of four helices and a two-stranded antiparallel β -sheet (Fig. 1a). The novel dimerization of IL-5 results in a large number of covalent, electrostatic and hydrophobic interactions between the two monomer chains.

Chemical modification or mutagenesis of the two conserved

cysteines leads to a biologically inactive protein^{15,16}. The symmetrical intermolecular disulphide links are unique to IL-5 when compared with the other cytokines. It could be argued on the basis of the crystal structure that the IL-5 monomer is unstable as many hydrophobic residues that are normally within the core of the dimer would be exposed to solvent (Fig. 2a). The IL-5 monomer and dimer have a total surface area of $9,300 \text{ \AA}^2$ and $11,500 \text{ \AA}^2$, respectively. This implies that an extremely large proportion of the monomer surface area, about $7,000 \text{ \AA}^2$, is at the interface of the two monomer chains. In addition to the covalent disulphide links, there are several other interactions between the two polypeptide chains. Arginine 32 to Val 35 ($\beta 1$) form an antiparallel β -sheet with residues Glu 89 to Arg 92 ($\beta 2'$) of the other monomer (Fig. 2a, b). The side chains of $\beta 1$ lie mostly buried within the helices, whereas the $\beta 2'$ residues are all solvent-accessible and hydrophilic. Immediately after residue 92 is a five-turn α -helix (helix D') that forms many interdigitating hydrophobic interactions with helices A-C from the other monomer chain (Fig. 2c). Three leucine residues from helix D' direct the packing into helices A-C: Leu 97, Leu 100 and Leu 104.

Even more surprising than the dimer configuration is that the eventual structural fold for the two domains of the dimer is homologous to the known crystal and NMR structures of granulocyte macrophage³ and macrophage⁴ colony-stimulating factors (GM-CSF, M-CSF), interleukins-2 and -4 (refs 5, 6), and similar to human⁷ and porcine⁸ growth hormones (hGH and pGH) (Fig. 2d), although these proteins do not share any

FIG. 1 a, Tube drawing of IL-5 that shows the novel dimer configuration. Each chain is shown in two colour schemes. One chain is dark yellow for an α -helix, lighter yellow for loop, and green for a β -strand. The other chain is white for an α -helix, purple for a loop, and light blue for a β -strand. Three different tube radii are used to denote helix, strand, and loop. The viewing direction is along the dimer 2-fold axis. The structure can be envisioned as two helical-bundle domains related by a 2-fold axis. An individual domain contains the GM-CSF fold: a bundle of four α -helices with two over-connecting loops. The β -sheet packs along the long axis of the 4- α -helix bundle on opposite sides of the dimer. Helices A and D' of both chains make up the surface facing the reader; helices B and C make up the opposite surface with the dimer 2-fold axis normal to both surfaces. Superpositioning of the two monomer chains (residues 7-109) results in an r.m.s. difference in backbone atoms (C α , C, O, N) and all atoms of 0.7 $\text{\AA}$ and 1.1 $\text{\AA}$, respectively. The figure was prepared with the program RASTER3D. b, C α stereo diagram of one IL-5 monomer shown in red, with every tenth residue labelled in green.



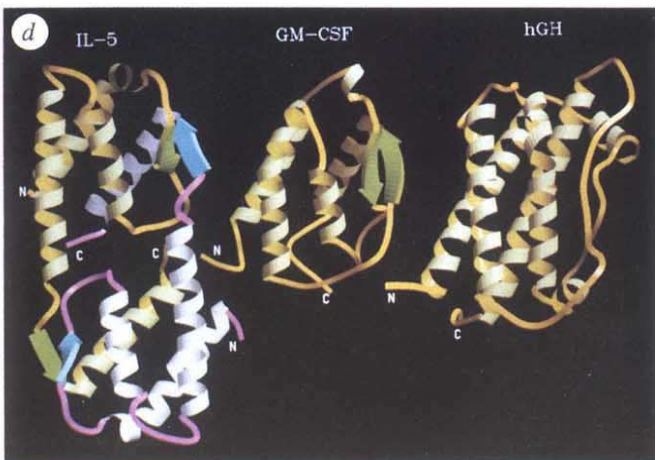
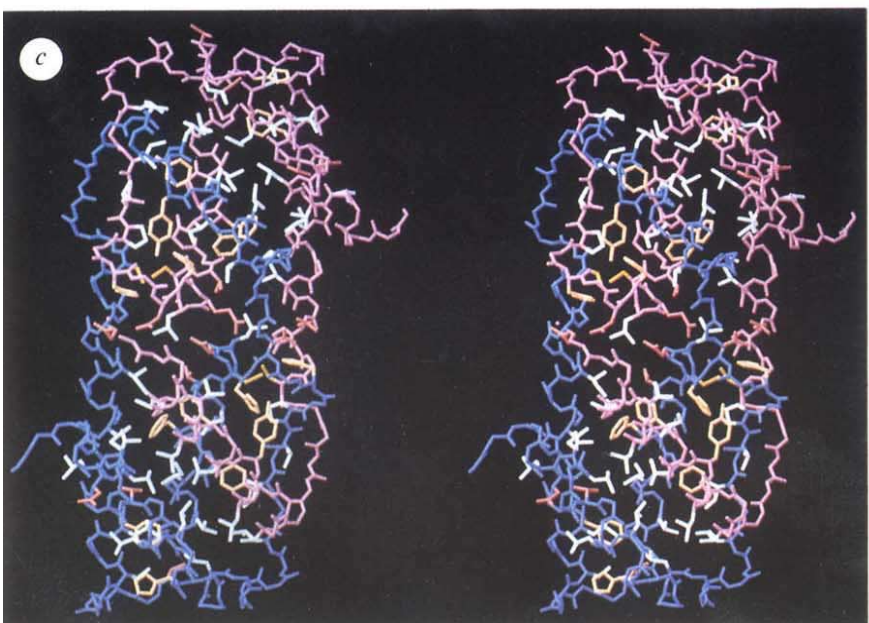
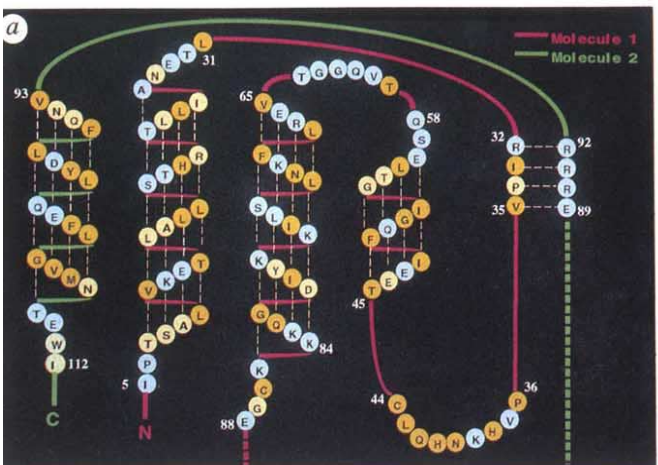
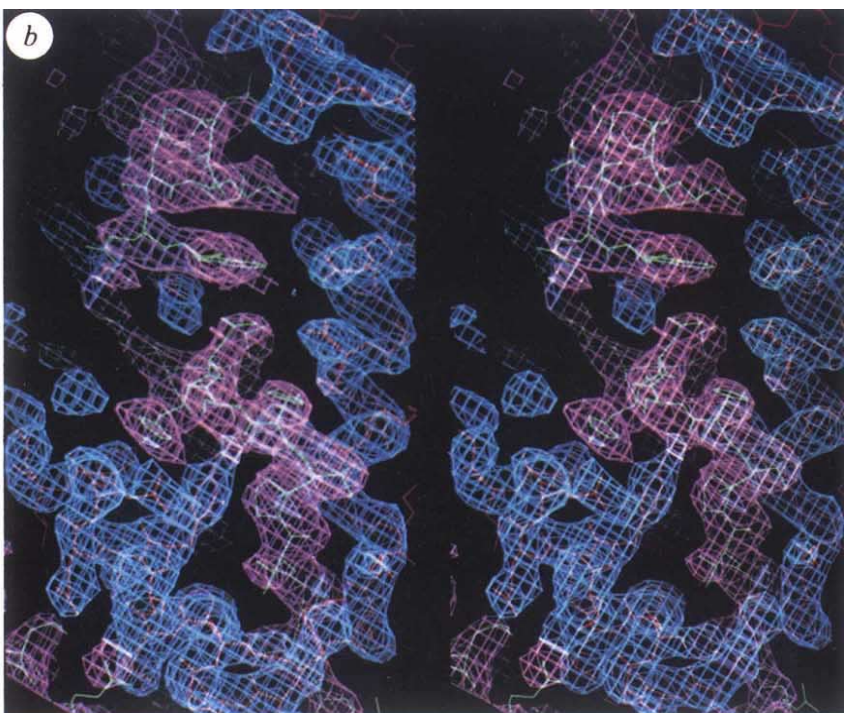


FIG. 2 *a*, Schematic representation is shown for one IL-5 domain. Amino acids are indicated by their one-letter code. An IL-5 domain is made up of one monomer chain shown in red and the other monomer chain in green. The colour code for the individual amino acids reflects side-chain solvent-accessible area: A: buried residues in orange ($A < 20 \text{ \AA}^2$); intermediate exposed residues in light yellow ($20 \text{ \AA}^2 < A < 55 \text{ \AA}^2$); exposed residues in light blue ($A > 60 \text{ \AA}^2$). Glycines are included in this analysis but Gly 87 and Gly 105 would have exposed side chains if they existed. The thin dashed white lines show backbone hydrogen bonds contributing to the secondary structure. *b*, Stereo view of the MIR electron density map at $3.0 \text{ \AA}$ resolution. The blue electron density contains the region within $1.7 \text{ \AA}$ of an atom in one monomer, and the purple electron density is similarly defined for the other chain. The electron density is contoured at 1σ . *c*, Stereo view of IL-5 with a polyglycine model shown in dark blue and purple for each chain. Side chains for those residues involved in internal molecular packing are coloured by residue class (A, P, V, I, L, M are white; S, T, N, Q are shown in red; H, F, Y are pale yellow; and C is bright yellow). *d*, Comparison of the three-dimensional structures of IL-5, GM-CSF and hGH. All three molecules are shown in roughly the same orientation of the helical bundles. Figure was prepared with the program MOLSCRIPT³⁴.

TABLE 1 Diffraction data and phasing statistics

Dataset	Resolution (Å)	Unique reflections	R_{merge}^* (%)	$R_{\text{scale}}^\dagger$ (%)	Number of sites	Phasing power ‡
Native	2.4	8,934	6.5			
K ₂ PtCl ₆	4.0	1,782	6.6	18.4	1	1.56
NaAuCl ₄	3.0	3,896	6.9	13.2	3	1.38
K ₃ IrCl ₆	3.5	2,860	5.9	14.7	1	1.40
K ₃ IrCl ₆ · Sel-met§	3.0	3,734	9.0	16.4	4	1.78
Sel-met§	3.5	2,928	8.2	8.6	2	2.00
Crystallographic refinement						
Resolution (Å)	R ($I > 0$)	R ($I > 2\sigma$)	Δ bond (Å)	Δ Angle (°)		
(8.0–2.4)	0.228 (8,880)	0.212 (8,456)	0.015	3.1		

The IL-5 crystal structure was determined to 3.0 Å using multiple isomorphous replacement (MIR) data collected on five different heavy-atom derivatives, including two crystals of selenomethionyl (Sel-met) IL-5. Crystals of native IL-5 and the sel-met mutant were in the space group C2 with unit cell dimensions of $a=122.1$ Å, $b=36.11$ Å, $c=56.42$ Å, $\beta=98.59^\circ$. Diffraction data were collected on a Siemens multiwire area detector and evaluated using the program XENGEN²⁸. Most of the crystallographic computations were performed with the program package PHASES²⁹. Heavy-atom sites were determined by inspection of Harker sections of difference Patterson maps and the use of the automated searching procedure in the program HASSP³⁰. The sel-met sites could be found from a difference Patterson map and gave good initial single isomorphous replacement (SIR) phase information, as ascertained from difference Fourier electron density maps calculated with difference coefficients with the other heavy-atom derivatives. The initial electron density map at 3.0 Å resolution had a mean figure of merit of 0.62 before solvent flattening, and a 0.81 mean figure of merit after solvent flattening. This initial electron density map allowed a polyaniline model to be fitted with four helices and two loops for two molecules in the asymmetric unit using the program FRODO³¹. An improved mask of the dimer molecule was calculated from these crude models and further improved manually using the program MAPVIEW²⁹ modified for an Evans and Sutherland workstation. The non-crystallographic twofold axis was found from these models and refined with the initial MIR electron density map. Electron density maps were averaged in combination with solvent flattening. This resulted in a relatively unambiguous electron density map for tracing the backbone and identifying many of the residues in the helix regions. Only residues 31–37 and 87–95 were unclear in terms of identifying the amino-acid registration, but the backbone density was clearly visible. These two regions were initially left out of the crystallographic refinement and stringent non-crystallographic symmetry restraints were applied. This partial model was refined with X-PLOR³² and combined with the original MIR phase information at 3.0 Å resolution. Residues 5 to 112 of both monomers in the IL-5 dimer were then fitted to the resulting electron density map. Several more cycles of simulated-annealing refinement and manual model rebuilding resulted in an R -factor of 21.3% and a 'free' R -factor³⁵ of 36.9% at 2.4 Å resolution. Certain regions of the map were omitted and refined using simulated annealing to confirm the tracing, with particular attention to the region of crossover between the two monomer chains. The final model has 33 water molecules, residues 5 to 112, and atomic temperature factors ranging from 10.0 to 52.4 Å² ($\sigma=8.4$) for main-chain protein atoms (residues 5–108 and 9–112) and 18.3 to 51.1 Å² for water atoms. The only temperature factors for the main chain atoms that exceed 52.4 Å² are at the N and C terminus. The current R -factor is 21.2% ($I > 2\sigma$) for all of the measured data between 8.0 and 2.4 Å (62% complete in the high-resolution shell between 2.49 Å and 2.40 Å). The root-mean-square (r.m.s.) deviations from ideal bond lengths and bond angles are 0.015 Å and 3.1°, respectively. Only two residues ϕ , ψ angles (Gly 62 and Gly 63) lie outside of the 'allowed' regions of the Ramachandran plot. IL-5 coordinates will be deposited in the Bookhaven Protein Data Bank.

* $R_{\text{merge}} = \sum |I_{\text{avg}} - I_i| / \sum I_i$.

† $R_{\text{scale}} = \sum |F_{\text{native}} - F_{\text{derivative}}| / \sum F_{\text{native}}$.

‡ Phasing power is the mean value of the heavy-atom structure factor amplitude divided by the residual lack of closure error.

§ Selenomethionyl protein crystal (Sel-met).

significant sequence similarity^{17,18}. This cytokine fold shows two important differences when compared with other bundles of four α -helices¹⁹. First, the IL-5 helices A and D' and helices B and C are at an angle of $\sim 20^\circ$ to one another, whereas the pairs of helices (A, D' and B, C) are $\sim 40^\circ$ relative to one another (Fig. 1b). This 40° interhelical angle is atypical of most bundles of four α -helices. Second, the up-up-down-down topology of the four helices for IL-5 and the other cytokines is different from the more frequently observed up-down-up-down connectivity. This cytokine fold must be important in receptor binding, and it is now the most common topology found among cytokines.

There are some functional similarities between IL-5, GM-CSF, IL-2 and IL-4. All of their receptors belong to the type I cytokine receptor family²⁰, and GM-CSF and IL-5 share an identical receptor β -subunit²¹. Furthermore, these cytokines have four exons and three introns, with a similar number of codons in each exon²². IL-5 is distinct from these cytokines in its absolute requirement for dimerization.

So far the receptor binding site on IL-5 is not well defined. The murine and human IL-5 share 70% sequence similarity but display some species-specific activity. Chimaeric proteins in which the last 36 residues are replaced with the species of interest shared activity²³. In this domain there are only eight residues that differ between human (h) and mouse (m) with only three non-conservative amino-acid substitutions: h-Gly 81 to m-Arg 81, h-Lys 84 to m-Glu 84, and h-Asn 94 to m-Arg 94. Glycine 81 and Lys 84 are part of the C-terminal end of the third helix, and amino-acid substitutions at these two positions would probably produce little structural perturbation. Conversely, Asn 94

lies in the N-terminal end of the last helix, and substitution here could lengthen the last loop connection. The role of the C-terminal residues in the biological activity of human IL-5 is also inferred from experiments involving CNBr digestion of the C-terminal eight amino acids and oxidation of Met 107 (ref. 24). None of these experiments seems to affect dimer formation. The two C-terminal helices of the dimer that are implicated in receptor recognition are proximal to one another (Fig. 1a). Thus there are two nearly identical surfaces of the dimer on the same face of the molecule. The exposed residues on helix D or D' that could interact with the receptor are Asp 98, Gln 101, Glu 102, Gly 105, and Asn 108.

The four- α -helix bundle with a 'double overhand' topology is a common fold for many cytokines. In fact, IL-5 is a dramatic evolutionary example of the diversity with which proteins can achieve a functional structure. Are there many other cytokines that bind to this receptor family and have the same polypeptide fold? In an attempt to answer this question, we aligned several other cytokine sequences, whose tertiary structure was unknown, with the sequences of known IL-5-like structures. As there is little primary sequence homology between any of these cytokines^{17,18}, we used a method that incorporates secondary structure predictions into the alignment procedure^{25,26} in an effort to increase the sensitivity (Table 2). The amino-acid sequences and predicted secondary structures of IL-6, IL-7, IL-10, IL-11, stem cell factor and erythropoietin align particularly well with one or more of the known structures, suggesting that these cytokines adopt the four- α -helix bundle topology, whereas IL-3 and leukaemia inhibitory factor give lower scores, so that no prediction can be made. This commonality among

TABLE 2 Cytokine tertiary structure predictions

Query sequence*	Tertiary structure†	Number of residues used in alignment	Most similar structure	Normalized Z score‡
IL-1 β	β -Barrel	153	M-CSF	0.04
IL-2	4 α -2 β	133	IL-4	3.25
IL-3	Unknown	133	GH	2.66
IL-4	4 α -2 β	129	IL-5	4.95
IL-5	4 α -2 β	115	IL-4	4.38
IL-6	Unknown	183	M-CSF	3.58
IL-7	Unknown	129	IL-2	4.72
IL-8	1 α -4 β	72	IL-4	1.33
IL-10	Unknown	161	IL-2	3.71
IL-11	Unknown	182	IL-5	4.34
GM-CSF	4 α -2 β	127	M-CSF	3.27
G-CSF	Unknown	174	GM-CSF	2.29
M-CSF	4 α -2 β	150	GM-CSF	3.18
GH	4 α	191	IL-2	1.79
LIF	Unknown	179	GH	2.32
SCF	Unknown	164	M-CSF	6.35
EPO	Unknown	166	GM-CSF	3.55

Abbreviations: LIF, leukaemia inhibitory factor; SCF, stem cell factor; EPO, erythropoietin. The amino-acid sequences and predicted secondary structures of several cytokines were aligned with the sequences and known secondary structures of IL-2, IL-4, IL-5, GM-CSF, M-CSF, hGH. Gaps and amino-acid substitutions were scored according to previous recommendations³³. The predicted secondary structures²⁵ were represented in terms of calculated probabilities of α , β and coil secondary structures, and each residue was given an additional score equal to the probability of adopting the secondary structure implied by the alignment. The joint alignment of sequence and secondary structure was carried out using an extension of the Needleman-Wunsch algorithm²⁶. The score, S, for each alignment was normalized by carrying out 141 additional alignments with sequences of equal length selected randomly from the Brookhaven protein databank. These normalized scores, Z, provide evidence for similar tertiary structures when greater than 3.5. As a further control, 126 sequences from the Brookhaven Protein Databank were aligned with the cytokines of known tertiary structure. The Z values were below 3.0 in all cases other than the 4- α -helix bundles and globins (data not shown). Alignments were also carried out between several of the known cytokine structures. IL-2, IL-4, IL-5, M-CSF and GM-CSF gave higher scores, correctly predicting their polypeptide fold, and GH gave an intermediate score that made prediction impossible. IL-1 β and IL-8 resulted in very low scores, which agree with their known tertiary structures.

* Human sequences were used in all cases except for mouse IL-7.

† 4 α -2 β refers to the topology 4- α -helix bundle with two over-connecting loops. 1 α -4 β refers to a structure with one α -helix and a four-stranded β -sheet.

‡ The normalized score is $Z = (S - \mu) / \sigma$, where μ and σ are the mean and standard deviation of scores obtained with randomly selected sequences of equal length. The bold-faced scores identify the cytokines of unknown tertiary structure most likely to have the topology of the 4- α -helix bundle with two over-connecting loops.

so many cytokine tertiary structures has important implications for receptor recognition and for antagonist and agonist designs. Recently, a potent antagonist of the human growth hormone was developed from a single-site amino-acid mutation based on the receptor/ligand crystal structure²⁷. This type of approach for IL-5 and these other structurally related cytokines will help in understanding their signal transduction pathways and in developing new therapeutic agents for diseases that involve this important class of proteins. □

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The dTAF_{II}80 subunit of *Drosophila* TFIID contains β -transducin repeats

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A KEY component of the RNA polymerase II transcriptional apparatus, TFIID, is a multi-protein complex containing the TATA box-binding protein (TBP) and at least seven tightly associated factors (TAFs)^{1,2}. Although the functions of most TFIID subunits are unknown, it is clear that TAFs are not necessary for basal activity but that one or more are required for regulated transcription, and so behave as coactivators^{1–4}. The presence of multiple subunits indicates that there is an intricate assembly process and that TAFs may be responsible for other activities. We have described the properties of the subunit dTAF_{II}110, which can interact directly with the transcriptional activator Sp1 (ref. 5). In addition, the largest subunit, dTAF_{II}250, binds directly to TBP and links other TAFs to the complex⁶. Here we describe the cloning, expression and partial characterization of the *Drosophila* TAF of M_r 80,000, dTAF_{II}80. Sequence analysis reveals that dTAF_{II}80 contains several copies of the WD40 (β -transducin) repeat⁷. Moreover, dTAF_{II}80 shares extended sequence similarity with an *Arabidopsis* gene, *COPI*, which encodes a putative transcription factor that is thought to regulate development⁸. We have expressed recombinant dTAF_{II}80 and begun to characterize its interaction with other members of the TFIID complex. Purified recombinant dTAF_{II}80 is unable to bind TBP directly or to interact strongly with the C-terminal domain of dTAF_{II}250 (Δ N250). Instead, dTAF_{II}80 is only able to recognize and interact with a higher-order complex containing TBP, Δ N250, 110 and 60. These findings suggest the formation of TFIID may require an ordered assembly of the TAFs, some of which bind directly to TBP and others that

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are tethered to the complex as a result of specific TAF/TAF interactions.

First, we generated antibodies against dTAF_{II}80 that immunoprecipitate a TBP/TAF complex with a subunit composition indistinguishable from that obtained with anti-TBP antibodies (Fig. 1a). We then used polyclonal antisera to isolate a complementary DNA clone, λ D4, encoding a protein that cross-reacted with various monoclonal antibodies directed against dTAF_{II}-80. Next, we used λ D4 to screen a *Drosophila* embryo λ gt10 library to identify potential full-length cDNA clones. The longest cDNA isolated, λ D5, contained a polyadenylated 3' end

and appeared to be nearly full-length when compared to endogenous messenger RNA identified by RNA blot analysis (data not shown). The complete DNA sequence of λ D5 (2,373 nucleotides) was determined, and the deduced amino-acid sequence revealed a protein of 704 residues (Fig. 2a). The protein has a calculated M_r of 79,300 (79.3K) and an estimated isoelectric point of 5.8. Six peptide sequences generated by tryptic digestion of purified endogenous dTAF_{II}80 (Fig. 1b) are contained within the open reading frame of λ D5 (Fig. 2a), thus confirming the identity of the clone. We mapped the dTAF_{II}80 gene to position 47C5-6 by chromosome *in situ* hybridization.

FIG. 1 a, Antibodies directed against dTAF_{II}80 immunoprecipitate the same TFIIID complex as anti-TBP antibodies. Complexes were immunoprecipitated from ~200 μ g TFIIID fraction using either a monoclonal antibody (3D10) directed against dTAF_{II}80 or an antibody (25B4) directed against dTBP. Proteins were fractionated on a 7.5% SDS-polyacrylamide gel and visualized by silver staining. Six dTAFs and TBP are indicated (under these conditions dTAF_{II}30 comigrates with the light chains of the antibodies and is therefore not visible). 'G' indicates a protein present in the hybridoma supernatant that binds protein G nonspecifically. b, Immunopurified TAFs were resolved using high-performance liquid chromatography (HPLC), and ~5 μ g purified dTAF_{II}80 was obtained. These fractions were then digested with trypsin and the resulting peptides (shown in Fig. 2a) sequenced. IN, input material for HPLC fractionation.

METHODS. Immunoprecipitation of the *Drosophila* TFIIID complex with anti-dTBP antibodies has been described⁴. Anti-dTAF_{II}80 monoclonal antibodies were derived by a method similar to that described for anti-dTAF_{II}110 hybridomas⁵; immunoprecipitations were done as described, except that protein G-Sepharose (Pharmacia) was used instead of protein A. Methods for large-scale TAF preparations, HPLC fractionation, and proteolytic digestion have been described⁵, except that the protein was cleaved in 100 mM ammonium bicarbonate and 2 M urea containing 150 ng trypsin.

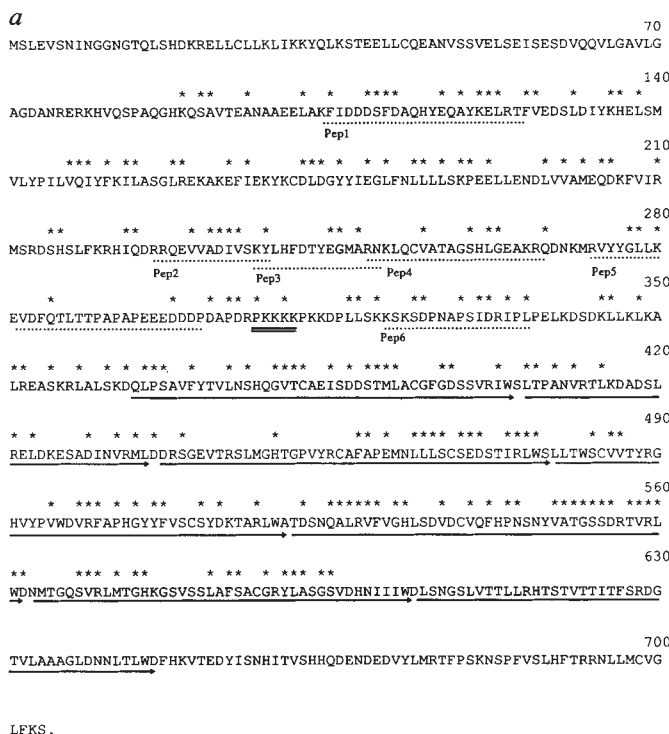
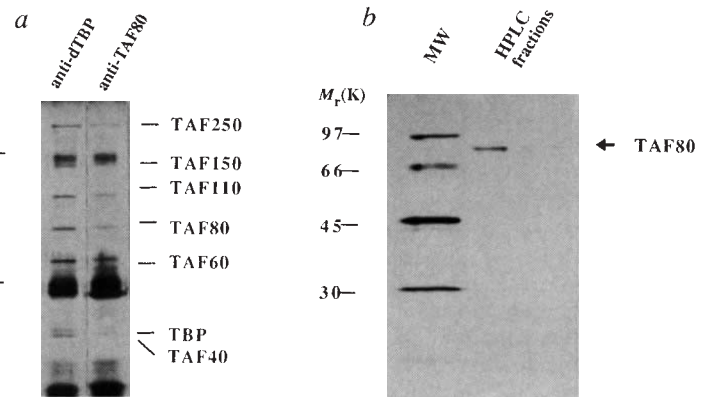
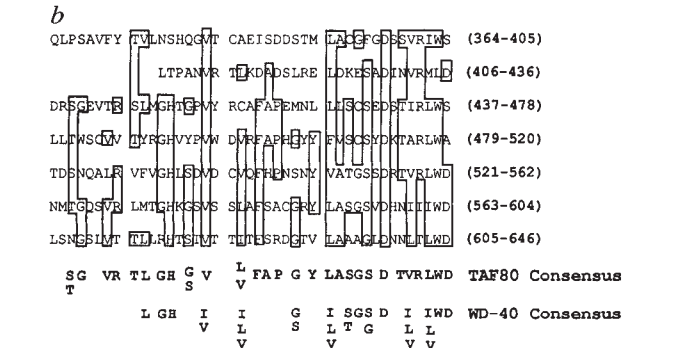


FIG. 2 Amino-acid sequence of dTAF_{II}80 and comparison with WD40 repeats and *COP1*. a, Deduced amino-acid sequence derived from the dTAF_{II}80 cDNA. The broken underline and bold numbers indicate the six tryptic peptides obtained as for Fig. 1b. The WD40 repeat region and a putative nuclear localization signal are indicated by a solid single and double underline, respectively. Residues that are similar or identical in the *Arabidopsis* gene *COP1* (ref. 8) are indicated by asterisks. Similarity rules are: G=A=S,



P=A=S, S=T=A, D=E=Q=N, K=R=H, V=I=L=M=F, F=Y. b, The WD40 repeats of dTAF_{II}80. Amino-acid residue numbers are shown on the right. Blocked regions indicate residues conserved within the dTAF_{II}80 protein, and conservation of a given amino acid on at least three occasions yields the consensus shown directly below the alignment. A consensus for WD40 repeats¹⁴ was derived from the alignment of bovine transducin, *PRP4*, *Enhancer of Split*, *CDC4* and *STE4*.

METHODS. dTAF_{II}80 was cloned using two independent approaches. A mouse polyclonal serum raised against the *Drosophila* TFIIID complex was used to screen a λ gt11 expression library constructed from 9–12-hour embryos¹⁹ as before⁵. A partial cDNA isolated in this screen was then used as a template in polymerase chain reactions (PCR) to confirm that it encoded dTAF_{II}80 with primers corresponding to peptides 1 and 4. The 0.5-kilobase (kb) fragment from an *EcoRI* digest of this λ phage (containing the two peptides, as judged by PCR) was labelled using Klenow polymerase and random hexamer priming (Amersham). About 10^6 recombinant phage from a 3–9-hour *Drosophila* embryo cDNA library²⁰ were screened as described²¹. Of 22 positives obtained in the primary screen, 10 were selected for re-screening: 7 were positive in this secondary screen. Four of these were apparently related by restriction enzyme analysis, and the longest cDNA was subcloned into pBluescript KS (Stratagene), resulting in plasmid pBS-80K. Both strands of the insert were sequenced by the dideoxy method after constructing exonuclease III deletions.

Analysis of the predicted primary structure of dTAF_{II}80 reveals several interesting features. A database search uncovered a strong similarity between the C-terminal portion of dTAF_{II}80 and a family of proteins that includes the β -subunit of G proteins⁹⁻¹¹, CDC4 (refs 12, 13), PRP4 (refs 14, 15) and TUP1 (ref. 16). These proteins have diverse biological roles in the cell, including signal transduction, cell-cycle regulation, splicing, and transcriptional repression, respectively. They all contain between four and eight consecutive 40-amino-acid repeating units known as β -transducin or WD40 repeats⁷. A DIAGON plot analysis revealed that dTAF_{II}80 contains seven tandem WD40 repeats (Fig. 2b). A consensus for this repeating unit has been derived¹⁴ and closely matches the repeats in dTAF_{II}80 (Fig. 2b). Although the function of these repeats is unknown, a role in protein-protein interactions seems most plausible^{7,14}. Several partners that interact with WD40 proteins have been reported^{14,17}, and in many cases they contain TPR repeats¹⁸. It will be of interest to test transcription factors and other components of the transcriptional machinery for potential interfaces and TPR repeats that could interact with dTAF_{II}80.

A particularly striking sequence similarity was observed between dTAF_{II}80 and the *Arabidopsis* gene *COPI*, which is important during the development of this plant. *COPI* is thought to encode a putative transcription factor of 75K which contains four WD40 repeats and a zinc-finger⁸. The two proteins share 22% identity and 45% similarity within the WD40 repeats. Moreover, this conservation is not restricted to those residues within the WD40 consensus, but is distributed throughout the N-terminal region (Fig. 2a), indicating that these two genes may be distantly related.

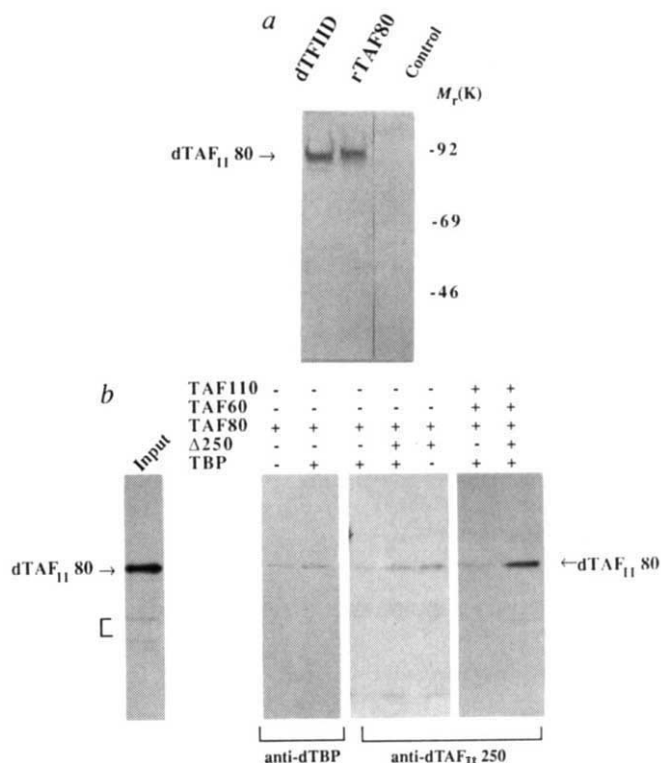
A pattern emerging of WD40 repeats in transcription factors suggests that they may be involved in repression mechanisms. The TUP1-SSN6 complex is a general repressor involved in diverse functions such as carbon metabolism¹⁷ and cell-type

specification in yeast. This repressor complex can be recruited to various target promoters by sequence-specific DNA-binding proteins¹⁸ and thus is likely to participate in several types of protein-protein interaction. Genetic evidence suggests that the *Arabidopsis COPI* product also acts as a repressor during development⁸. On the basis of this similarity, dTAF_{II}80 might be a component of TFIID that is involved in mediating repression.

To begin a characterization of the biochemical properties of dTAF_{II}80, we have produced this protein in a baculovirus expression system. The electrophoretic mobility of the recombinant protein is indistinguishable from the endogenous product, and western blot analysis confirms that the recombinant protein crossreacts efficiently with monoclonal antibodies directed against dTAF_{II}80 (Fig. 3a). As a first step towards characterizing the function of dTAF_{II}80 and its relationship with the other components of the TFIID complex, we have attempted to assemble this subunit *in vitro* into the TFIID complex. First, we asked whether dTAF_{II}80 can interact directly with TBP and found that this subunit does not bind TBP, as determined by co-immunoprecipitation (Fig. 3b). This result suggests that additional TAFs are required for dTAF_{II}80 to associate with TBP. We have shown that the C-terminal domain (Δ N250) of the largest subunit of TFIID, dTAF_{II}250, can specifically interact with dTBP and dTAF_{II}110 (ref. 6). We therefore tested the ability of dTAF_{II}80 to interact with Δ N250, either in the presence or absence of dTBP. Unlike dTAF_{II}110, we found that dTAF_{II}80 was unable to interact strongly with either Δ N250, or with a TBP/ Δ N250 partial complex. Coimmunoprecipitation experiments with partially denatured TAFs also suggested that endogenous dTAF_{II}250 and dTAF_{II}80 do not interact in a stable manner by themselves (data not shown). By contrast, if we preassemble a complex containing TBP, Δ N250, dTAF_{II}110, and dTAF_{II}60, then we detect efficient incorporation of

FIG. 3 Recombinant dTAF_{II}80 has the same apparent M_r as the endogenous protein and associates with a TBP-TAF complex. **a**, Representative western blot of an SDS-8% polyacrylamide gel showing that cloned dTAF_{II}80 overexpressed in insect cells migrates with the same apparent mobility as endogenous dTAF_{II}80 from *Drosophila* embryos. The right lane contains a control Sf9 extract expressing an unrelated gene. Arrow indicates the position of dTAF_{II}80. **b**, Autoradiograms showing that dTAF_{II}80 does not interact directly with dTBP but can associate with it by interactions with other TAFs. Various combinations of recombinant dTBP, dTAF_{II}60, dTAF_{II}110 and the C-terminal domain of dTAF_{II}250 (Δ N250) were incubated with ³⁵S-labelled dTAF_{II}80. The resulting protein complexes were then immunoprecipitated with either anti-TBP or anti-dTAF_{II}250 antibodies. Arrow indicates the position of dTAF_{II}80, and brackets indicate incomplete translation or degradation products of this protein as judged by immunoprecipitations with antibodies against this TAF. An amount equivalent to 20% of the input for the binding reactions was loaded in the first lane (IN).

METHODS. A dTAF_{II}80 baculovirus expression vector was constructed by creating an *NdeI* site initiator methionine of the cDNA in plasmid pBS-80K using a PCR-based strategy. The fragment containing the complete coding region of dTAF_{II}80 was subcloned into the baculovirus expression vector pVL1393 (Pharminogen). The dTAF_{II}80 *in vitro* transcription/translation vector was constructed by subcloning the dTAF_{II}80 coding region into pT β STOP (ref. 22). dTBP, dTAF_{II}110 (ref. 5), dTAF_{II}60, and dTAF_{II} Δ N250 (R.O.J.W., unpublished results; ref. 6) were produced from recombinant baculovirus-infected Sf9 cells. Extracts were prepared by sonicating infected Sf9 cells in 0.1 HEMG-NDP (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 12.5 mM MgCl₂, 10% glycerol, 0.1 M KCl, 0.1% NP-40, 1.5 mM DTT, 0.1 mM PMSF and 5 μ g ml⁻¹ leupeptin). This sonicate was centrifuged and the supernatant partially purified by passing it over Q- and/or S-Sepharose (Pharmacia) columns equilibrated in 0.1 HEMG-NDP; the protein eluted in a 0.2–0.4 M KCl step. dTAF_{II}110 was found in the flow-through fraction of both columns. dTAF_{II} Δ N250 was partially purified by chromatography over a Q-Sepharose column equilibrated in 0.1 HEMG-NDP, when the protein eluted in a 0.2–0.4 M KCl step. dTBP was purified over S-Sepharose (equilibrated in 0.1 HEMG-NDP) by elution with a 0.2–0.4 M KCl step. The per cent purity of each recombinant protein was as follows: dTBP, 80%; dTAF_{II}110, 1–5%; dTAF_{II}60 and Δ N250, less than 1%. For co-immunoprecipitation, protein G beads were preloaded with monoclonal antibody 30H9 directed against dTAF_{II}250 (ref. 6) or with



anti-dTBP polyclonal antibody, and incubated at 4 °C successively with various combinations of partially purified dTAF_{II} Δ N250, dTBP, dTAF_{II}110 and dTAF_{II}60 for 1 h per step, with occasional mixing. Complexes were briefly washed with 0.1 HEMG-NDP between additions. ³⁵S-labelled dTAF_{II}80 was then added, allowed to bind for 1 h at 22 °C and then washed extensively with 0.1 HEMG-NDP. The proteins bound to the beads were analysed by SDS-PAGE and autoradiography.

dTAF_{II}80 into the partial complex (Fig. 3b). The cloning, characterization and expression of the gene encoding dTAF_{II}60 will be described elsewhere. These results suggest that dTAF_{II}80 will bind efficiently and become an integral part of the TFIID complex only when several other TAFs are preassembled. Our findings also indicate that there are essential TAF/TAF interactions that must take place during the assembly of the TFIID complex. Future studies will be aimed at dissecting the role of different TAFs in the assembly and function of the TFIID complex. Experiments addressing potential interactions of dTAF_{II}60 and dTAF_{II}110 with dTAF_{II}80 are currently in progress. It should be possible to test whether WD40 repeats are involved in either TAF/TAF, or TAF/activator/repressor interactions. □

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RNA editing as a source of genetic variation

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KINETOPLASTID RNA editing alters mitochondrial RNA transcripts by addition and deletion of uridine residues¹, producing open reading frames that may be twice as long as the original RNA². Although the *COIII* gene encoding cytochrome *c* oxidase subunit III in *Trypanosoma brucei* is edited along its entire length², the presumably homologous genes in two related trypanosomes, *Leishmania tarentolae* and *Crithidia fasciculata*, are only modestly edited at their 5' ends¹. We used a comparative approach to investigate the evolution of an edited gene and to determine how well editing creates conserved protein sequences. As RNA editing probably involves the pairing of several guide RNA molecules with the messenger RNA^{3,4}, we expected the edited proteins to be resistant to evolutionary change. Here we report that RNA editing is extensive in the mitochondria of four species of the insect parasite *Herpetomonas*, which is possibly an evolutionary precursor of *T. brucei* and *L. tarentolae*⁵, and the discovery that RNA editing is a novel source of frameshift mutations over evolutionary time. The edited proteins accumulate mutations nearly twice as rapidly as the unedited versions.

We used a polymerase chain reaction (PCR) strategy which exploits the tandem arrangement of genes on the maxicircle to amplify the *COIII* gene from four species of *Herpetomonas*: *H. mariadeanei*, *H. samuelpessoai*, *H. muscarum muscarum* and *H.*

TABLE 1 Per cent pairwise similarity between species for DNA, edited RNA and protein

Predicted protein sequence similarity						
	Hsa	Hme	Hmm	Hma	Tbr	Lta
<i>H. samuelpessoai</i>						
<i>H. megaseliae</i>	91					
<i>H. m. muscarum</i>	91	99				
<i>H. mariadeanei</i>	77	77	78			
<i>T. brucei</i>	74	77	77	72		
<i>L. tarentolae</i>	72	72	73	68	71	
<i>C. fasciculata</i>	75	76	76	70	73	90

Edited RNA sequence similarity						
	Hsa	Hme	Hmm	Hma	Tbr	Lta
<i>H. samuelpessoai</i>						
<i>H. megaseliae</i>	92					
<i>H. m. muscarum</i>	92	99				
<i>H. mariadeanei</i>	84	85	85			
<i>T. brucei</i>	81	83	82	80		
<i>L. tarentolae</i>	75	75	75	74	77	
<i>C. fasciculata</i>	74	74	75	73	75	86

DNA sequence similarity, aligned with edited RNA as in Fig. 1

	Hsa	Hme	Hmm	Hma
<i>H. samuelpessoai</i>				
<i>H. megaseliae</i>	92			
<i>H. m. muscarum</i>	92	99		
<i>H. mariadeanei</i>	91	89	89	
<i>T. brucei</i>	82	83	83	82

DNA sequence similarity, in compressed form as in Fig. 3

	Hsa	Hme	Hmm	Hma
<i>H. samuelpessoai</i>				
<i>H. megaseliae</i>	93			
<i>H. m. muscarum</i>	93	99		
<i>H. mariadeanei</i>	86	85	84	
<i>T. brucei</i>	82	81	80	81

Per cent amino-acid replacements due to frameshifts

	Hsa	Hme	Hmm	Hma	Tbr	Lta
<i>H. samuelpessoai</i>						
<i>H. megaseliae</i>	62					
<i>H. m. muscarum</i>	63	100				
<i>H. mariadeanei</i>	49	46	45			
<i>T. brucei</i>	44	40	41	52		
<i>L. tarentolae</i>	37	31	30	25	32	
<i>C. fasciculata</i>	35	31	30	33	28	17

Sequence comparisons are for the coding region. Abbreviations as in Fig. 1 legend.

megaseliae. *COIII* is flanked at its 3' end by the well conserved cytochrome *b* sequence, providing a downstream PCR primer, and at its 5' end by *MURF3*, which is less conserved and extensively edited *T. brucei*⁶. We therefore chose an upstream PCR primer from within the *COIII* DNA sequence of *T. brucei*². Direct sequencing of these PCR products⁷ gave the unedited *COIII* sequences corresponding to the last 230 bases of these genes (Fig. 3).

We determined the completely edited mRNA sequence for each of the four species of *Herpetomonas* by direct sequencing of RNA PCR products using primers based on the *T. brucei* complementary DNA sequence^{2,8} and also by one-sided anchor PCR^{9,10}, extending either to the 5' end of the mRNA or to the 3' untranslated region including the poly(A) tail, followed by cloning and sequencing. As some of the 5' anchor-PCR products from *H. mariadeanei*, *H. m. muscarum* and *H. megaseliae* were partially edited at the 5' end (data not shown), we could identify PCR primers to amplify the rest of the mitochondrial *COIII* gene in these three species.

Figure 1 shows that there is extensive editing in the *COIII* transcript from each of the four species of *Herpetomonas*. Uridine additions (and rare deletions) create 90–93% of the 288 amino-acid codons. Uridine insertions also appear in the poly(A) tails, as in other pan-edited genes². PCR amplification

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FIG. 1. Alignment of *COIII* DNA and edited RNA sequences for *H. samuel-
pessoai* (Hsa), *H. megaseliae* (Hme), *H. m. muscarum* (Hmm), *H. mariadeanei*
(Hma), *T. brucei* (Tbr), *L. tarentolae* (Lta)^{1,12} and *C. fasciculata* (Cfa)^{1,13}. DNA
sequences in upper case; uridines in mRNA that are added by RNA editing
in lower case; encoded thymidines deleted from the mRNA indicated by
gaps. Frameshifted nucleotides are highlighted. The start and stop codons
are underlined and the corresponding positions on the amino-acid sequences
(Fig. 2) are numbered below the *C. fasciculata* sequence. Editing adds 548
uridines at 198 sites and deletes 27 uridines at seven sites in *H. mariadeanei*,
creating 59% of the 867-nucleotide open reading frame. Similarly, editing
adds 569 uridines in *H. megaseliae* and 572 uridines in *H. m. muscarum*.

at 208 identical sites and deletes 12 uridines at seven sites in *H. megaseliae* and 13 uridines at eight sites in *H. m. muscarum*, creating 60% of the coding sequence. These last two closely related sequences differ from each other only at one uridine deletion, one silent A → G transition at position 510, one frameshift at position 109, which was a polymorphism in *H. m. muscarum*, and two insertions of extra uridines in the 5' and 3' untranslated regions. In the region in which we can compare both the genomic DNA and edited RNA for *H. samuelpeessoai*, 310 new uridines appear at 122 sites and six disappear at three sites: uridine insertions create 61% of the coding sequence. Between Hme, Hmm and Hsa, 35, 37 frameshifts or suppressor frameshifts occur in the coding region; 50, 50, 52 occur between Hsa, Hmm,

with primers corresponding to the edited mRNA failed to detect any genomic copy that matched the edited sequence for each case (data not shown).

Table 1 compares the degrees of sequence similarity between species at the DNA, RNA and predicted protein levels. *C. fasciculata* and *L. tarentolae*, which are edited in only 6% of the amino-acid codons, show a higher degree of sequence conservation at the amino-acid level (90%) than at the mRNA level (86%), as would normally be expected with silent changes outweighing replacements. But the highest degree of sequence conservation for the pan-edited genes is at the RNA and DNA level, in spite of massive sequence deletion. The level of predicted protein similarity decreases significantly in pairwise comparisons between *Herpetomonas* and *T. brucei* or within *Herpetomonas*, owing to frameshift mutations that are introduced by changes in editing which are compensated by suppressor frameshift mutations at a nearby editing site, producing about half of the amino-acid replacements, which cluster in the frameshifted regions (Fig. 2). The unusual amino-acid substitutions leucine (UUG) to cysteine (UGU) to valine (GUU) occur frequently as a result of a local frameshift. In different species, editing often produces different mRNA sequences from the same unedited DNA sequences (Fig. 1), providing an additional source of genetic novelty. Figure 3 shows an alignment of the compressed cryptic DNA sequences which reveals sites that are conserved in the DNA but shifted to another location in the mRNA. We propose that each of these frameshifts in the edited mRNA is the result of two compensatory mutations in the guide RNAs, small maxicircle or minicircle transcripts that appear to mediate editing by base-pairing with specific regions of the edited transcript, allowing some G·U base-pairs. Complete editing proceeds 3' to 5' and requires a set of overlapping guide RNAs. Editing by each guide RNA creates an anchor sequence for binding the next guide RNA^{3,4}. Thus RNA editing is a surprisingly inefficient mechanism for conserving amino-acid sequence.

The requirement for base-pairing imposes additional restrictions on the mRNA sequence such that synonymous substitutions may not be neutral. We do observe a nearly threefold lower rate of synonymous changes in the edited genes, either for the entire reading frame or just in the regions where there are no frameshifts (Fig. 1). Although the rate of A-to-G transitions is not significantly different for edited or unedited genes, presumably reflecting the ability of a U in the guide RNA to pair with either A or G, the U-to-C transitions are completely suppressed. Furthermore, only a fraction of the gene is actually encoded in the DNA. The number and location of DNA-encoded uridines that are deleted by editing also vary greatly among all of the species. Between species, the 5' and 3' untranslated regions are even more divergent in both the unedited and edited sequence, suggesting that constraints are relaxed outside the coding region.

Do the fully edited transcripts correspond to actual proteins? One difficulty with our interpretation is the lack of an in-frame

AUG in the fully edited mRNAs from *H. mariadeane*, *H. m. muscarum* and *H. megaseliae*, although a conserved AG exists at the same DNA position in every case (Fig. 3). Editing creates an in-frame AUG in the other kinetoplastid species, but inserts more than one uridine in each of these three examples, perhaps to make the non-canonical initiation codon UUG¹¹. The predicted N-terminal amino-acid sequences are very highly conserved in all of the species, and editing creates a conserved stop codon and predicted amino-acid sequences of the same length (Fig. 2), strengthening the view that the genes are functional. We are confident that the sequences in Fig. 1 are the fully edited transcripts, because we sequenced several clones from each 5' anchor PCR, and we used direct PCR sequencing to obtain most of the sequences, including the 5' ends. There are also two examples in *L. tarentolae* of transcripts that do not contain AUG initiation codons¹, including one that is edited.

Do the edited proteins have a fast clock? The ratio of protein sequence distances to the actual pairwise mitochondrial ribosomal DNA distances (ref. 5; and L.F.L. and W.G., manuscript in preparation) uses the changes in the mitochondrial ribosomal RNA genes as a measure of the evolutionary distances between species. Pairwise comparisons of these normalized rates of protein sequence divergence give values from 1.10 to 1.47 between species with extensive editing and divergence (1.17 between *H. samuelpessoai* and *H. m. muscarum*; 1.35, 1.47 between *H. samuelpessoai*, *H. m. muscarum* and *H. mariadeane*; 1.23, 1.10,

Hsa	OMFLFRVIFVGVSGVVFVLSLPAVVICVYVVLCCGFMICCGSFVFDVDMCF
Hme	OMFLFRVIFVGVSGVVFVLSLPAVVICVYVVLCCGFMICCGSFVFDVDMCF
Hmm	OMFLFRVIFVGVSGVVFVLSLPAVVICVYVVLCCGFMICCGSFVFDVDMCF
Hma	OMFLFRVIFVGVSGVVFVLSLPAVVICVYVVLCCGFMICCGSFVFDVDMCF
Tbr	OMFLFRVIFVGVSGVVFVLSLPAVVICVYVVLCCGFMICCGSFVFDVDMCF
Lta	OMFLFRVIFVGVSGVVFVLSLPAVVICVYVVLCCGFMICCGSFVFDVDMCF
Cfa	OMFLFRVIFVGVSGVVFVLSLPAVVICVYVVLCCGFMICCGSFVFDVDMCF

Hsa	50VFFLVCLLFCILLFCDFVDFLRGIFDFLTFIRCLQYCFIWFVISELVL
Hme	50VFFFGLLFCILLFCDFVDFLRGIFDFLTFIRCLQYCFIWFVISELVL
Hmm	50VFFFGLLFCILLFCDFVDFLRGIFDFLTFIRCLQYCFIWFVISELVL
Hma	50VFFFGLLFCILLFCDFVDFLRGIFDFLTFIRCLQYCFIWFVISELVL
Tbr	50VFFFGLLFCILLFCDFVDFLRGIFDFLTFIRCLQYCFIWFVISELVL
Lta	50VFFFGLLFCILLFCDFVDFLRGIFDFLTFIRCLQYCFIWFVISELVL
Cfa	50VFFFGLLFCILLFCDFVDFLRGIFDFLTFIRCLQYCFIWFVISELVL

Hsa	100FLTFVTFVGYCIFLCCFAFVFLPMLFCCLLVDYGFVYWFIDFLNL
Hme	100FMSEFTVVFYGVIFLCCFAFVFLPMLFCCLLVDYGFVYWFIDFLNL
Hmm	100FMSEFTVVFYGVIFLCCFAFVFLPMLFCCLLVDYGFVYWFIDFLNL
Hma	100FMSEFTVVFYGVIFLCCFAFVFLPMLFCCLLVDYGFVYWFIDFLNL
Tbr	100FMSEFTVVFYGVIFLCCFAFVFLPMLFCCLLVDYGFVYWFIDFLNL
Lta	100FMSEFTVVFYGVIFLCCFAFVFLPMLFCCLLVDYGFVYWFIDFLNL
Cfa	100FMSEFTVVFYGVIFLCCFAFVFLPMLFCCLLVDYGFVYWFIDFLNL

Hsa	150LINTFLFVSGLFCNFFLFCVWFVFFVVCVFLVWCGILFGFLFNQWLWE
Hme	150LINTFLFVSGLFCNFFLFCVWFVFFVVCVFLVWCGILFGFLFNQWLWE
Hmm	150LINTFLFVSGLFCNFFLFCVWFVFFVVCVFLVWCGILFGFLFNQWLWE
Hma	150LINTFLFVSGLFCNFFLFCVWFVFFVVCVFLVWCGILFGFLFNQWLWE
Tbr	150LINTFLFVSGLFCNFFLFCVWFVFFVVCVFLVWCGILFGFLFNQWLWE
Lta	150LINTFLFVSGLFCNFFLFCVWFVFFVVCVFLVWCGILFGFLFNQWLWE
Cfa	150LINTFLFVSGLFCNFFLFCVWFVFFVVCVFLVWCGILFGFLFNQWLWE

Hsa	200FALLFVTCSCGVFGSILFLIDLHFTHVLLGVFLFVFCRLFNFLCMT
Hme	200FALLFVTCSCGVFGSILFLIDLHFTHVLLGVFLFVFCRLFNFLCMT
Hmm	200FALLFVTCSCGVFGSILFLIDLHFTHVLLGVFLFVFCRLFNFLCMT
Hma	200FALLFVTCSCGVFGSILFLIDLHFTHVLLGVFLFVFCRLFNFLCMT
Tbr	200FALLFVTCSCGVFGSILFLIDLHFTHVLLGVFLFVFCRLFNFLCMT
Lta	200FALLFVTCSCGVFGSILFLIDLHFTHVLLGVFLFVFCRLFNFLCMT
Cfa	200FALLFVTCSCGVFGSILFLIDLHFTHVLLGVFLFVFCRLFNFLCMT

Hsa	250RFVFLYVVFYWHFVDCVWFLLRFVYFVFLVLCVYLCV term
Hme	250RFVFLYVVFYWHFVDCVWFLLRFVYFVFLVLCVYLCV term
Hmm	250RFVFLYVVFYWHFVDCVWFLLRFVYFVFLVLCVYLCV term
Hma	250RFVFLYVVFYWHFVDCVWFLLRFVYFVFLVLCVYLCV term
Tbr	250RFVFLYVVFYWHFVDCVWFLLRFVYFVFLVLCVYLCV term
Lta	250RFVFLYVVFYWHFVDCVWFLLRFVYFVFLVLCVYLCV term
Cfa	250RFVFLYVVFYWHFVDCVWFLLRFVYFVFLVLCVYLCV term

FIG. 2 Alignment of the predicted amino-acid sequences for seven kinetoplastid species. Positions conserved in all seven species are marked with an asterisk; conservative amino-acid replacements are marked with a cross.

Hme and Hma; 62 between Tbr and Hsa; 46, 48 between Hme, Hmm and Tbr; 64 between Tbr and Hma; and possibly 8 between Lta and Cfa. The GenBank accession numbers for these sequences are L10845–L10852. METHODS. PCR amplification from either total DNA or first strand cDNA prepared with random hexamers, a *NotI*-d(T)₁₈ anchor primer (Pharmacia) or 3'-specific primer and murine reverse transcriptase (Pharmacia) from total RNA¹⁴ was as described¹⁵. The sequences of RNA PCR primers are underlined; negative-strand primers are italicized. Single-stranded templates for direct sequencing¹⁵ were produced either by λ exonuclease digestion¹⁶ or by capture of a biotinylated strand onto streptavidin coated magnetic beads⁷. 5' anchor-PCR products, using G-tailed cDNA and 5' [CUA]₄CTCGAGAATT(C)₁₂(GAT) primer, and 3' anchor-PCR products^{9,10} were visible on an ethidium-bromide-stained agarose gel after nested PCR with an internal primer⁹ and the anchor¹⁰. Anchor-PCR products were gel-purified, cloned (T-vector, Novagen, and UDG cloning, BRL) and at least three clones were sequenced on both strands with *Taq* DNA polymerase (Promega fmol sequencing system).

Hme 0 AGATATATAAGGC-----GGA-GGGGAA
Hmn 0 CTAAATAGATATAAGGC-----GGA-GGGGAA
Hma 0 TGTAGACAGATAAACTAAAC-AT-TAAAGGC-----GGA-GGGGAA
Tbr 0 TTATTAGGATTGTTTAAATTAATTAATAAGGCTTTTGGGAAGGGG-A

Hme 22-----GTGGGACACCTGCT-CGGATTGAAGAAGAG-----GTGATGAGG
Hmn 28-----GTGGGACACCTGCT-CGGATTGAAGAAGAG-----GTGATGAGG
Hma 40TTTT-G-GGGAACGCTGCTTCGGATTG-AGGAGGATT-G-GA-GAAG
Tbr 48TTTTG-GGGACACC-GC--CAGA--GGAGGAGGTTTGTG-GA-AGAG

Hme 62---G---GAGGGGATAG---GT-GAGGGGGAATTTTGAAGGAAGAG
Hmn 68---G---GAGGGGATAG---GT-GAGGGGGAATTTTGAAGGAAGAG
Hma 83---G---GAGGGGGA-GG---GTGGGGGGGAGCTTT--AAGGAAGGAA
Tbr 89TTTGTGTTTGAAGGA-GGTTTGTG--AGGGGAGG-----GAGAGAGGGA

Hme 100CGGAGAGAAACGG-GCAGAGAGGGGA---G--GAACCTGGGAGCAACT-
Hmn 106CGGAGAGAAACGG-GCAGAGAGGGGA---G--GAACCTGGGAGCAACT-
Hma 119CGGAGAGAGACGG-GCAGAGAGGGAATTTG--GAG---GGGACAGCTT-
Tbr 131CGGAGAGAGAACGGACAGAGGAGGA---GTTGAG---GAAGCGGTTT

Hsa 0 GG
Hme 143-G---GGGAGAGGGGAGGCTTTTGGGCCAG---GGGAAG--GAAGGG
Hmn 148-G---GGGAGAGGGGAGGCTTTTGGGCCAG---GGGAAG--GAAGGG
Hma 162-GTTTGG-GAGAGGGGAGCTTTTGGGCCAATTTTGGGGGTTGAAGGG
Tbr 175TG---AAGGAGAGGGGAGGCTTTGGGCCA-AG---GGGAAG--GAAGGG

Hsa 2AGG--A--AGAGAAGAAAAACAAT--A---GAGGGG-GAAA-----GG
Hme 183AGG--ATTGAGAAGAAAAACAAT--A---GAGGGG-GAAA-----GG
Hmn 188AGG--ATTGAGAAGAAAAACAAT--A---GAGGGG-GAAA-----GG
Hma 210AGG--AT-AGAGAAGAAAAACAATTTG--GAGGGGTGAAG-----GG
Tbr 215AGGTTA--GAAAAAGAAAAACAATTTG--GAGGGG-GAAG-----GG

Hsa 32-----GGAGAGG---GGGAGGGGGAAG---GGAGGAATCCAGG-GAG
Hme 221-----GGAGAGG---GAGAGGGGGAAG---GGGGGA-CCAAG-GAG
Hmn 227-----GGAGAGG---GAGAGGGGGAAG---GGGGGA-CCAAG-GAG
Hma 249TTTT-GGAGAGATT-GGAGGGGGGAG---GAGAGAA-CATGG-GAG
Tbr 254TTTTGGAGGGTTTGGGAGAGAGGGGTTTGGGGA-CCAGATGAG

Hsa 72A--A---GCGGAGACAGATTTTGGG---GGGAGGATT-GAG-GCAA
Hme 261A--A---GCGGAGACAGATTTTGGG---GGGAGGATT-GAG-GCAA
Hmn 267A--A---GCGGAGACAGATTTTGGG---GGGAGGATT-GAG-GCAA
Hma 291A--A---G-GAGGACAGATT-TGGG---AGGCAGGATTGAA-ACAA
Tbr 303ATTGTTTGCAGAAACAA---GGGGTTTTTGGGCAAG---GAATACAA

Hsa 110C--TCAGGA-GGG-GGAGGGCGAAAGGAGGAACCTCG-GGAGGGGAGACG
Hme 297C--TCAGGA-GGG-GGAGGGCGAAAGGAGGAACCTCGGAGGGGAGACG
Hmn 303C--TCAGGA-GGG-GGAGGGCGAAAGGAGGAACCTCGGAGGGGAGACG
Hma 328C--CCAG-ATGGGAGAGAGCGAGGGGAGGAACACCTGGAGGGGAGACG
Tbr 346TTTGTCAG-A-GGGGGGAGAGCGGAAGGAGGAACACG-GGAGGGAAGACG

Hsa 155GA---GGGGGGCGAGAGAGAG---GGGAGG-G-AGAAGGAGGGGTGAGAA
Hme 343GA---GGGGGGCGAGAGAGAG---GGGAGGTTG-A-AA---GGGGGAAATT
Hmn 349GA---GGGGGGCGAGAGAGAG---GGGAGGTTG-A-AA---GGGGGAAATT
Hma 375GATT-GGGGAGCGAGAGAGGTTTGAAGG-G-A-AA---GGGGGGGAAT
Tbr 394GATTAGGAAGCGAGAGAGAG---GAGAGG-GGA-AA---GGG-----T

Hsa 197AAAAG-GTGTAGTGTG-TGGAAGAATAAA
Hme 383TAA---GTGT-GTGT-----AGAATATAGAAAAATAAA
Hmn 389TAA---GTGT-GTGT-----AGAATATAGAA
Hma 417TTAGGAGTGTAGT-GAG-TAGTATATAACAACTACTAAACAAAAACA
Tbr 429TTAGTGTGAATGAAGAGGTAGTTTGTAGGAAGTTAA

FIG. 3 Conservation of cryptic DNA sequences. The unedited *COIII* genes for *Herpetomonas* and *T. brucei* are aligned. The sequences are underlined of the primers used to amplify the 5' end of the gene and the DNA fragment described in ref. 2. We did not determine the 5' end of the *H. samuelpessoai* DNA sequence because none of the 5' anchor PCR products were partially edited at the 5' end. The Hsa sequence begins 17 bp downstream from the PCR and sequencing primer. The sequence of the negative-strand cytochrome b primer is 5'-biotin-CCTAACTAA(AT)CC(AT)AC(CA)CCATA-3'.

1.30 between *H. samuelpessoai*, *H. m. muscarum*, *H. mariadeane* and *T. brucei*), compared to a value of 0.69 between *L. tarentolae* and *C. fasciculata*. Thus the edited proteins evolve on average 84% faster (between 60% and 113% faster) than the 5'-edited versions because of frameshifting. We expected the requirement for multiple changes in the guide RNAs to inhibit the rate of amino-acid change for edited genes, as it does for synonymous changes. Instead, the extensively edited trypanosomatid mitochondrial genes appear to be driven in rapid genetic variation.

Note added in proof: The authors of ref. 12 suggest that the *L. tarentolae* COIII protein itself is unusually rapidly evolving, even among the other maxicircle encoded proteins. Thus the extensively edited COIII proteins probably evolve even faster than our calculation shows. □

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Mentation of the DNA repair defect in xeroderma pigmentosum group G cells by a human cDNA related to yeast *RAD2*

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DEFECTS in human DNA repair proteins can give rise to the autosomal recessive disorders xeroderma pigmentosum (XP) and Cockayne's syndrome (CS), sometimes even together¹⁻³. Seven XP and three CS complementation groups have been identified that are thought to be due to mutations in genes from the nucleotide excision repair pathway^{2,3}. Here we isolate frog and human complementary DNAs that encode proteins resembling RAD2, a protein involved in this pathway in yeast^{4,5}. Alignment of these three polypeptides, together with two other RAD2 related proteins^{6,7}, reveals that their conserved sequences are largely confined to two regions. Expression of the human cDNA *in vivo* restores to normal the sensitivity to ultraviolet light and unscheduled DNA synthesis of lymphoblastoid cells from XP group G, but not CS group A. The XP-G correcting protein XPGC is generated from a messenger RNA of ~4 kilobases that is present in normal amounts in the XP-G cell line.

Our interest in these repair defects has its origin in a third human disorder. Systemic lupus erythematosus (SLE) patients often generate antibodies against a protein called La that has been implicated in transcription termination by RNA polymerase III (ref. 8). To recover the frog homologue of the La protein, we screened a *Xenopus laevis* oocyte cDNA library in λ gt11 with an SLE patient serum having strong anti-La reactivity. This yielded a La cDNA⁹, but also a distinctly different clone with an incomplete open reading frame; several longer clones were then recovered by hybridization screening of a *X.laevis* oocyte cDNA library in λ gt10. The longest contains a 3.73-kilobase (kb) cDNA that potentially encodes an acidic protein (pI 4.8) of 1,196 amino acids (*M_r* 134,206). The predicted protein is 24.6% identical (37.5% similar) to *Saccharomyces cerevisiae* RAD2, a protein involved in nucleotide excision repair^{4,5}. This degree of relatedness is similar to that found between the human and yeast DNA repair proteins XPAC and RAD14 (refs 10, 11), suggesting that the protein may represent the frog homologue of RAD2.

Why did a C-terminal fragment of this protein react with the SLE serum if it is not obviously related to La? Possibly it shares with La a common antigenic determinant. Alternatively, because

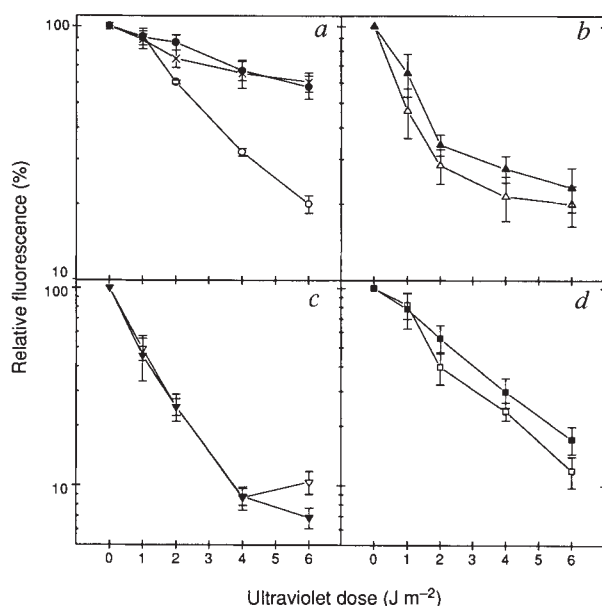
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TABLE 1 Unscheduled DNA synthesis in control and transfected XP-G cell lines

Cell line	Transfected plasmid DNA	Grains per nucleus
XP81	—	27.6 ± 1.3
XP83	—	1.7 ± 0.2
XP83	EBO-pLPP	1.1 ± 0.2
XP83	EBO-pLPP-hRAD2	28.8 ± 1.6

Cells were plated onto microscope slides, irradiated with 254 nm light (15 J m^{-2}) and then incubated at 37°C for 2 h with culture medium containing 10% serum, $10 \mu\text{Ci ml}^{-1}$ [methyl- ^3H]thymidine (25 Ci mmol^{-1} ; Amersham), and $2 \mu\text{M}$ fluorodeoxyuridine. After a 1-h chase with medium containing $100 \mu\text{M}$ thymidine and $10 \mu\text{M}$ deoxycytidine, cells were rinsed, fixed, autoradiographed and stained²⁶. Values show the mean number of grains per nucleus $\pm$ standard error of the mean from groups of 50 non-S-phase cells. Slides with non-irradiated cells were processed simultaneously and showed 1–3 grains per nucleus in all four cases.



Expression of the human RAD2-related protein in XP-G cells restores their ultraviolet sensitivity to normal levels, whereas the presence of the EBO-pLPP vector alone does not lead to any improvement (Fig. 2a). The complementation is specific for XP-G, as indicated by the lack of correction of XP-A or XP-C cells (Fig. 2b, c). These negative results are expected because XP-A complementing activity resides in a protein resembling RAD14 (refs 10, 11), and the XP-C defect is corrected by a hydrophilic protein related to RAD4 (ref. 18). The XP-G complementing protein fails to correct CS-A cells (Fig. 2d) and this protein is clearly different from the normal CS-C and CS-B gene products^{17,21}. Hence it is likely that those XP-G patients with CS symptoms^{2,23} belong to a new CS complementation group. This, considered together with the evidence for a CS/XP-D overlap²⁴, suggests the existence of at least five CS complementation groups.

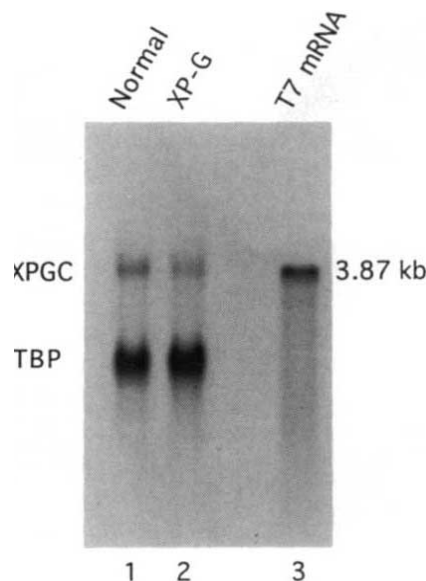
The XP-G complementation is independently corroborated

FIG. 2 Ultraviolet sensitivity of human lymphoblastoid cell lines transfected with EBO-pLPP vector (unfilled symbols) or EBO-pLPP-hRAD2 (filled symbols). a, Untransfected GM1953 cells (repair-proficient control, crosses), and XP83 transfectants (XP-G, circles); b, GM2246 (XP-C, triangles); c, GM2345 (XP-A, inverted triangles); d, GM2964 (CS-A, squares).

METHODS. The Epstein-Barr-virus-immortalized homozygote XP-G cell line XP83 was a gift from R. D. Wood; all other cell lines were from the Human Genetic Mutant Cell Repository (Coriell Institute, New Jersey). Cells were grown in suspension at 37°C in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal calf serum under 5% CO_2 . EBO-pLPP-hRAD2 was created by inserting a cDNA for the human RAD2-related protein, together with 67 bp of natural 5' leader, into the unique *NotI* site of the EBO-pLPP expression shuttle vector²². For transfection, cells were suspended at $10^7 \text{ cells ml}^{-1}$ in medium containing $400 \mu\text{g ml}^{-1}$ *Escherichia coli* transfer RNA and $20 \mu\text{g ml}^{-1}$ plasmid DNA, and were electroporated at 250 V, 960 μF . Transfectants were selected with increasing doses of hygromycin B (Calbiochem) up to $150\text{--}250 \mu\text{g ml}^{-1}$. Once selected, transfectants were suspended at $4 \times 10^6 \text{ cells ml}^{-1}$ in Hank's buffer and were irradiated for various times at 12 W cm^{-2} with a 254 nm lamp. For each UV fluence, two aliquots of $200\text{-}\mu\text{l}$ cell suspension were cultured with 1.4 ml medium in 24-well plates; 48 h later $500 \mu\text{l}$ aliquots were incubated for 0.5 h at 37°C with $1 \mu\text{g ml}^{-1}$ BCECF-AM (Molecular Probes). Cells were washed in $250 \mu\text{l}$ ice-cold Hank's buffer, 1% Triton X100 and the fluorescence of two $100 \mu\text{l}$ aliquots was measured in 96-well plates in a Millipore Cytofluor at excitation/emission wavelengths of 485/530 nm (ref. 30). Results are expressed as per cent of the fluorescence of non-irradiated cells. Error bars show the standard deviations of each group of 4 measurements.

FIG. 3 Northern blot analysis of poly(A)⁺ RNA from normal (lane 1) and XP83 (lane 2) lymphoblastoid cell lines, together with a length marker of synthetic mRNA (lane 3).

METHODS. Cells were lysed in guanidinium isothiocyanate, total RNA was isolated by CsCl step-gradient centrifugation, and poly(A)⁺ RNA was purified on oligo(dT)-cellulose columns. Samples ($5 \mu\text{g}$) were fractionated by electrophoresis through a 1% agarose-formaldehyde gel and transferred by capillarity to a nitrocellulose membrane. The membrane was first hybridized with a probe of the entire XPGC cDNA labelled by random priming and then with a similarly labelled probe from the 3' end of human TATA-binding protein cDNA²⁵.



by a second repair assay. A cell line (XPG81) derived from the obligate heterozygote mother of an XP-G patient²³ undergoes significant unscheduled DNA synthesis (Table 1). In contrast, XPG83 cells from the patient, whether untransfected or transfected with the EBO-pLPP vector alone, show very low levels of unscheduled DNA synthesis, which are comparable to those in non-irradiated cells. The heterozygote level is fully restored in XPG83 cells expressing the human RAD2-related protein (Table 1). Moreover, in an accompanying letter²³ strong evidence is reported that this protein can correct the DNA repair defect in extracts of the XPG83 and other XP-G cell lines. Given these positive results of *in vivo* and *in vitro* complementation, we refer to this XP-G correcting protein as XPGC, and to its gene as *XPGC*.

Northern blot analysis shows that an XPGC mRNA of ~4 kb is present in normal lymphoblastoid and XPG83 cells (Fig. 3). Longer autoradiographic exposures provide no evidence for alternatively spliced transcripts. The signal intensities relative to the ~2.2 kb TBF mRNA²⁵ suggest that there is no significant reduction in the steady-state level of XPGC mRNA in these XP-G cells. This is confirmed by RNase mapping, which indicates that ~10 copies of XPGC mRNA are present in both normal and XPG83 cells (data not shown). Hence the increased ultraviolet sensitivity of these XP-G cells is likely to be due to a subtle rather than gross alteration of a normally active *XPGC* allele.

The XPGC protein sequence yields few clues to its function but its *in vitro* activity²³ suggests that it does not regulate, at the transcription or translation levels, the activity of a protein involved in nucleotide excision repair. Instead, XPGC probably participates directly in the repair process, although a regulatory role as a result of protein modification cannot be excluded. The

isolation of its cDNA is an essential step towards understanding how this protein participates in this crucial DNA repair pathway and perhaps in other processes. □

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Identical defects in DNA repair in xeroderma pigmentosum group G and rodent ERCC group 5

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HUMANS with the complementation group G form of the inherited syndrome xeroderma pigmentosum (XP) are hypersensitive to solar ultraviolet light because of a defect in nucleotide-excision repair of DNA^{1–4}. Some individuals are also affected with Cockayne's syndrome, and have neurological abnormalities. Here we report that the DNA repair deficiency of XP-G cell extracts can be corrected by addition of protein fractions from normal cells. Repair proficiency can also be restored by mixing XP-G cell extracts with extracts from different repair-defective cell lines, with one exception. Extracts from cells representing group 5 of a set of ultraviolet-sensitive rodent mutants fail to complement XP-G extracts. XP-G and group 5 correcting activities co-elute after ~1,000-fold purification from HeLa cells. An antibody directed against a recombinant fragment of the XP-G complementing protein (XPGC) inhibits excision repair by normal cell extracts, and activity can be restored with an XP-G/group 5 complementing fraction. These data strongly suggest that the XPGC and group 5 correcting (ERCC5) proteins are identical.

The repair defect in XP-G cells can be alleviated by microinjecting them with normal cell protein extracts^{5,6}, but the correcting factor has not been identified. To investigate the nature of the deficiency, we made use of the observation that XP-G cell extracts show poor repair of ultraviolet-irradiated DNA *in vitro*^{7,8}. We first asked whether protein fractions from normal

TABLE 1 Purification of XP-G and ERCC-5 complementing activity from HeLa cells

Fraction	Purification step	Volume (ml)	Protein (mg)	Specific activity (U µg ⁻¹)
1	HeLa S100 extract	250	5,500	—
2	Phosphocellulose/ ammonium sulphate	390	1,209	0.3
3	AcA34 gel filtration	150	255	0.9
4	Hydroxyapatite	12	56.4	1.4
5	DNA cellulose*	25	0.5	~100–400

* Fraction 4 (1 mg) was used for the double-stranded DNA/cellulose step. Specific activity was calculated as described (ref. 27) and could not be determined reliably for the crude extract. Allowing for a 3 to 4-fold enrichment by phosphocellulose chromatography and ammonium sulphate precipitation, the complementing activity was purified ~1,000 to 3,000-fold from the crude extract.

cells could restore the ability of XP-G cell extracts to perform DNA repair synthesis. HeLa cell extract was chromatographed on phosphocellulose, concentrated protein from the active fraction applied to a gel filtration column, and the activity of eluted material determined (Fig. 1a). XP-G correcting activity eluted with a relative molecular mass (*M_r*) of 260K with respect to the markers. The ability to restore repair to an XP-G cell extract *in vitro* in this way indicates that the correcting activity is a protein with a relatively direct function in repair. The complementation occurs *in vitro* in the absence of transcription, translation or DNA replication, although this does not preclude an additional role for the protein in one of these processes.

We previously found that complementation of repair can be achieved by mixing cell extracts from certain different repair complementation groups with one another^{7,9}. This approach

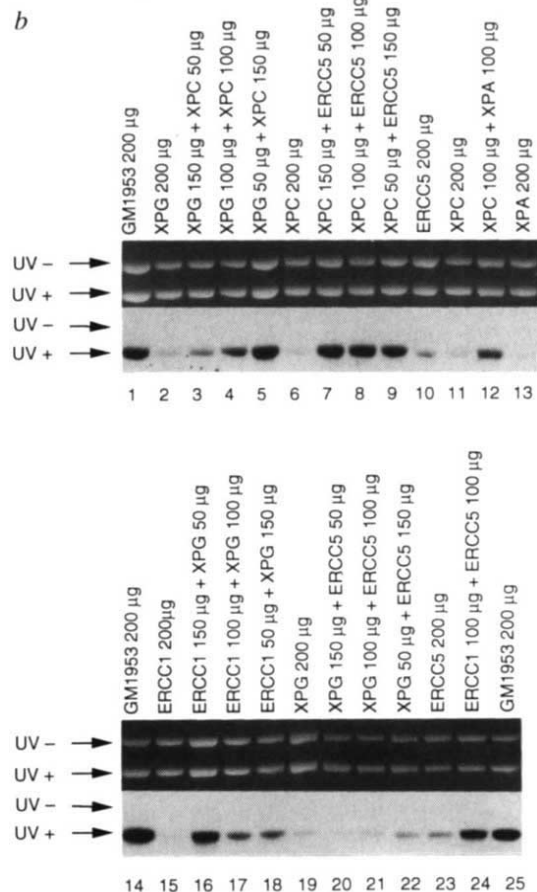
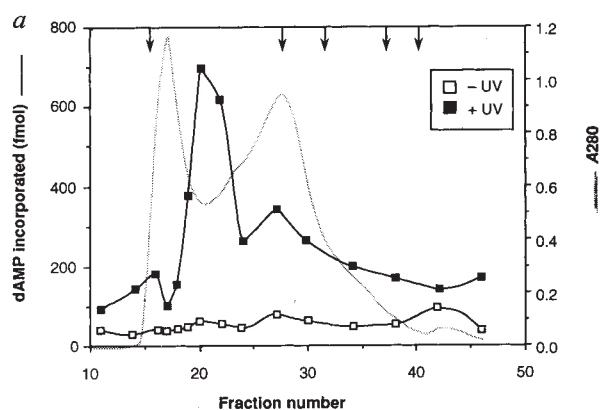


FIG. 1 *a*, XP-G complementing activity from HeLa cell extracts. Samples of fractions (6 μ l) eluted from a gel filtration column (A_{280} , stippled line) were mixed with 150 μ g whole cell extract protein from XPG83 cells to assay for complementation of nucleotide excision repair activity²⁷ with a mixture of ultraviolet (UV)-irradiated (filled squares) and unirradiated DNA (open squares). Arrows indicate (from left to right) the elution positions of blue dextran (void volume), alcohol dehydrogenase (M_r = 150K), bovine serum albumin (66K), carbonic anhydrase (29K), and cytochrome *c* (12.4K). *b*, Mixing cell extracts from different repair complementation groups to assess complementation of DNA repair synthesis. Lanes 1, 14 and 25, GM1953 normal cell extract. Lanes 2–6 mixtures of XP-G (XPG83) and XP-C (GM2249) cell extracts; lanes 6–10, mixtures of XP-C and ERCC5 (UV135) extracts; lanes 11–13, mixtures of XP-C and XP-A (GM2250) extracts; lanes 15–19, mixtures of XP-G and ERCC1 (43-3B) extracts; lanes 19–23, mixtures of XP-G and ERCC5 extracts; lanes 23–24, mixtures of ERCC1 and ERCC5 extracts.

METHODS. *a*, Frozen cell pellets from a 130-litre culture of HeLa cells were thawed, disrupted by homogenization and extracted with buffer A (25 mM HEPES-KOH, pH 7.8, 1 mM EDTA, 0.01% NP-40, 10% glycerol, 0.1 mM PMSF) containing 0.4 M NaCl. After centrifugation for 2 h at 100,000g the supernatant was dialysed against buffer A containing 100 mM KCl. The clarified dialysate was loaded onto an 800-ml phosphocellulose column (Whatman P11) pre-equilibrated in buffer A, and eluted with buffer containing increasing concentrations of KCl. Protein eluting between 0.1 and 0.4 M KCl (390 ml, 3.2 mg ml⁻¹) contained the bulk of XP-G complementing activity. This was concentrated by precipitation with (NH₄)₂SO₄ to 70% saturation, dialysed against buffer A containing 0.4 M KCl, and applied to a column (2.5 $\times$ 90 cm)

was used to check for the ability of extracts from other repair mutants to complement XP-G extract. Upon mixing extract from the XP-G cell line XPG83 with repair-defective extracts from other XP groups, reaction mixtures with normal repair activity were obtained. An example is shown in Fig. 1*b* (lanes 2–6), where XP-G and XP-C extracts were mixed together. Such a result was anticipated because cell fusion experiments strongly indicate that XP-G is genetically distinct from the other XP groups^{1,2}. Other ultraviolet-sensitive mutants of mammalian cells exist, however. Of particular interest is a set of mutants isolated from rodent cells that have been assigned to multiple complementation groups^{10,11}. Human genes (denoted *ERCC* genes) that can correct the distinct repair defects of five groups of these mutants have been isolated, and in two cases the *ERCC* genes correspond to XP complementing genes^{12,13}. The sequences for genes or proteins that complement groups 4 and 5, or the less studied groups 7–11, have not been reported, although cosmid clones have been isolated that can alleviate the group 5 defect¹⁴. We investigated whether mutants of one of these groups might correspond to XP-G.

Extracts prepared from cell line UV135 (representing rodent complementation group 5) were combined with extracts from XP cells of groups A, B, C, D and F. (XP group E cells have only a moderate defect in DNA repair, and suitable extracts could not be prepared from them.) In each case *in vitro* com-

plementation of repair synthesis was achieved (Fig. 1*b*, and data not shown). Exceptionally, however, mixtures of UV135 extract with extract from the XPG83 cell line did not restore repair (Fig. 1*b*, lanes 19–23). The UV135 extract likewise failed to complement extracts from the SV40-immortalized XP-G fibroblast cell lines XPG415A (ref. 15) and XP3BRSV15 (ref. 16) (data not shown). As a further control, XP-G extract was mixed with extract from cells of other rodent complementation groups and found to complement extracts from group 1 (Fig. 1*b*, lanes 15–19), as well as repair-defective extracts from groups 2, 3 and 4 (not shown).

The XP-G complementing activity from HeLa cells (Fig. 1*a*) was further purified and tested for its ability to complement group 5 extracts. During chromatography on hydroxyapatite, XP-G complementing activity eluted in 200 mM phosphate, in the same fraction as group 5 complementing activity (Fig. 2*a*). At this stage, the protein preparation contained only complementing activity for XP-G and rodent group 5 extracts and could not complement extracts from other tested XP or rodent repair groups (Fig. 2*b*, and data not shown). The active fractions from the hydroxyapatite column were loaded onto a column of double-stranded DNA cellulose, and the column was washed with 0.1 M KCl and then with 1.0 M KCl. The XP-G and rodent group 5 activities again co-purified, eluting in the 1.0 M KCl fraction (Table 1). These mixing and biochemical copurification

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FIG. 2 Copurification of XPG and ERCC5 complementing activities by hydroxyapatite chromatography. *a*, Active fractions from the gel filtration column (Fig. 1*a*) were pooled (150 ml, 1.7 mg ml⁻¹) and loaded onto a 30-ml hydroxyapatite column in buffer B (25 mM HEPES-KOH, pH 7.8, 0.2 mM EDTA, 0.4 M KCl, 5% glycerol, 2 mM DTT, 0.1 mM PMSF) containing 1 mM potassium phosphate. The flow-through was collected and the column was progressively eluted with buffer B containing 25 mM, 60 mM, 200 mM and 500 mM potassium phosphate. Peak fractions from each elution were pooled and dialysed against buffer A containing 100 mM KCl. An aliquot (6 µl) of each fraction was added to 150 µg protein from XP-G (XPG83) or ERCC5 (UV135) extracts for the *in vitro* repair assay. Quantification of repair synthesis in UV-irradiated and unirradiated DNA is shown. The background level of repair is reproducibly higher for UV135 extracts than for XPG83 extracts (Compare Fig. 1*b*, lanes 19 and 23). *b*, A 6-µl aliquot of the pooled and dialysed fractions from the 200 mM phosphate elution (fraction 4, F4) was added to 150 µg cell extract as indicated, and repair synthesis was measured as described in the legend to Fig. 1. Cell lines with the GM prefix were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, New Jersey, USA). The cell line XPG83 was established by Epstein-Barr virus immortalization of lymphocytes from XP-G patient XP125LO⁴. The 43-3B line was derived as described²⁸. Other lines were provided by the investigators who originally isolated them: UV41 and UV135 (ref. 10); XPG415A (ref. 15); XP3BR-SV15 (ref. 16).

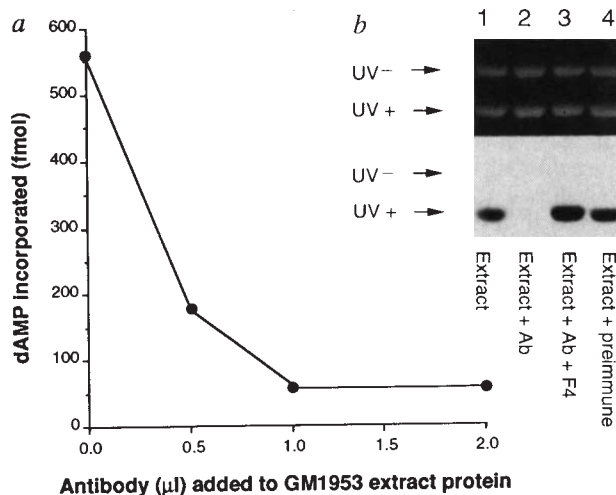
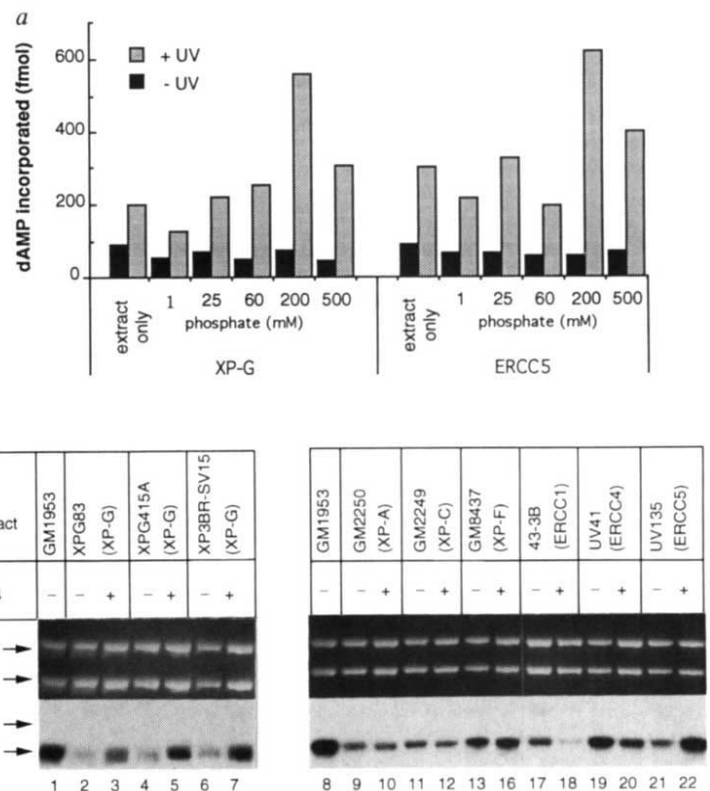


FIG. 3 *a*, Neutralization of DNA repair synthesis in normal GM1953 cell extracts by an antibody raised against a fragment of XPGC (corresponding to the evolutionarily conserved N-terminal 108 residues)¹⁷. Preimmune serum had no effect. *b*, Restoration of repair activity by addition of purified XPGC complementing activity (200 mM phosphate fraction; Fig. 2*a*). In each reaction, 150 µg GM1953 cell extract protein (normal extract) was preincubated with immunopurified antibody (Ab) for 30 min at 37 °C. A mixture of UV-irradiated and unirradiated plasmids was then added with the other components of the repair reaction mixture and incubated for 3 h at 30 °C followed by analysis as described in the legend to Fig. 1.

results strongly suggest that the XPGC and ERCC5 proteins are identical.

In an accompanying letter, the isolation of a cDNA that can correct the DNA repair defect of XPG83 cells is reported¹⁷. To examine any relationship between this cDNA and our XP-G complementing activity, antibodies recognizing an N-terminal polypeptide of XPGC were incubated with repair-proficient GM1953 cell extracts. Addition of the antibody progressively

reduced repair synthesis to a level comparable to an XP-G cell extract (Fig. 3), whereas preimmune serum had no effect. Moreover, the hydroxyapatite fraction containing XP-G/group 5 correcting activity restored the repair proficiency of the extract (Fig. 3*b*), providing a strong argument that the XPGC gene encodes the activity in our purified protein fraction. In addition, the XPGC antibody reveals a band of the expected size on an immunoblot of fraction 4 (not shown). The elution position of XP-G complementing activity during gel filtration chromatography (Fig. 1*a*) may indicate that the 133K XPGC polypeptide forms a homodimer, or is associated in the extracts with one or more proteins. One possibility is that XPGC and ERCC5 are distinct but closely related proteins which co-purify. However, we have mapped the XPGC gene to human chromosome 13q32-33 by *in situ* hybridization (S. Samec *et al.*, manuscript in preparation). The defective gene in UV135 (group 5) cells has been localized to chromosome 13q14-34 by somatic cell hybridization and chromosome breakpoint analysis¹⁸, and gene fragments that can complement the ERCC5 defect map to 13q33 (refs 19, 20). Thus the chromosomal locations of XPGC and ERCC5 also coincide.

We have used *in vitro* complementation with soluble cell extracts to demonstrate that a human XP complementation group corresponds to a rodent repair complementation group. Three groups of ultraviolet-sensitive rodent mutants have now been shown to correspond to XP defects—ERCC2/XPDC (ref. 12), ERCC3/XPBC (ref. 13), ERCC5/XPGC (this study)—and one to a separate Cockayne's syndrome (CS) defect, ERCC6/CSBC (ref. 21). Both the XP-B and XP-D groups include individuals affected with Cockayne's syndrome (refs 13, 22). Cells from three individuals²³ with ultraviolet sensitivity and the characteristic neural defects of Cockayne's syndrome were assigned to the XP-G complementation group by somatic cell fusion experiments (W. Vermeulen and J. H. J. Hoeijmakers, personal communication). The occurrence of patients with symptoms of Cockayne's syndrome in all three of these XP groups emphasizes the genetic overlap between the two diseases.

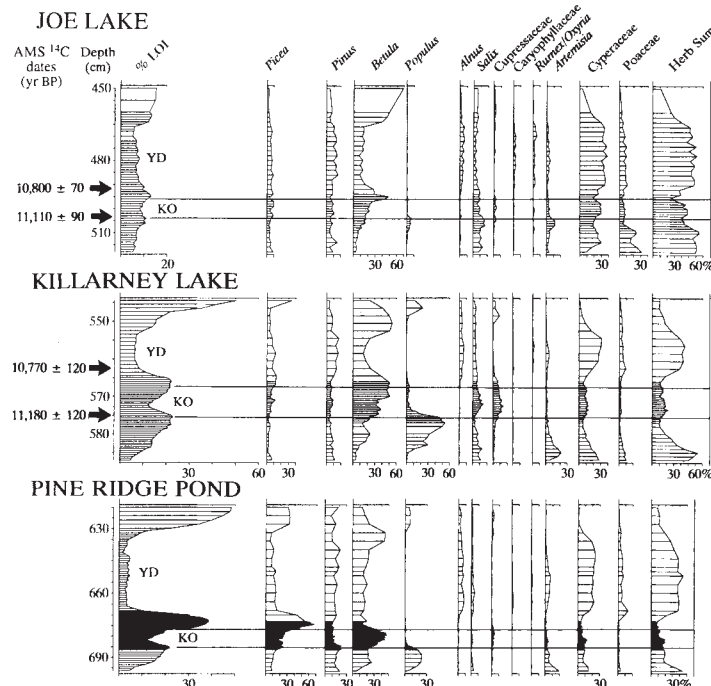
Neurological symptoms could arise from defects in *XP/ERCC/CS* genes in at least two ways. Unrepaired DNA damage might be particularly deleterious to critical gene expression in nervous tissue and lead to progressive neurological deterioration²⁴. Alternatively, developmental abnormalities might arise if an *ERCC/XP/CS* gene product functioned in both DNA repair and gene expression. This may indeed be the case for *ERCC3/XPBC/CS-C* (refs 25, 26).

Two XP groups remain for which genes have not been assigned (XP-E and XP-F), and any relationship between these and the set of ultraviolet-sensitive rodent complementation groups could be investigated by a similar approach. In this way a more complete picture can be drawn of the activities and genes involved in nucleotide excision repair which will facilitate mechanistic dissection of the process. □

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CORRECTION

Flightless bird from the Cretaceous of Mongolia

Perle Altangerel, Mark A. Norell, Luis M. Chiappe & James M. Clark

Nature **362**, 623–628 (1993)

It has been brought to our attention by Ben Creisler that the genus name *Mononychus* used to refer to a new flightless Cretaceous bird in our letter to *Nature* is preoccupied by the coleopteran *Mononychus* Schueppel 1824 (ref. 1). We therefore propose the replacement *Mononykus* for this genus whose type species is *Mononykus olecranus*.

- Schueppel, J. F. in *Insectorum species novae aut minus cognitae, descriptionibus illustratae* Vol. 1 *Coleoptera* (ed. Germar, E. F.) (Halle, 1824).

ERRATUM

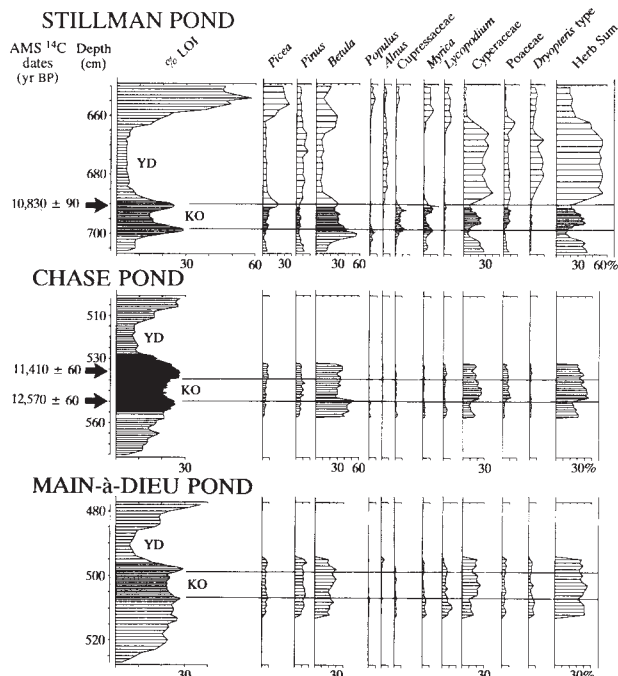
A previously unrecognized late-glacial cold event in eastern North America

André J. Levesque, Francis E. Mayle, Ian R. Walker & Les C. Wynar

Nature **361**, 623–626 (1993)

IN the printed version of Fig. 2 in this letter, several of the ¹⁴C dates shown by arrows were omitted. The correct version is shown below, together with the legend.

FIG. 2 Pollen percentage diagrams from six sites in Maritime Canada. The thick continuous horizontal lines indicate the boundaries of the KO based on the LOI curves, as discussed in the text. YD, Younger Dryas. Each horizontal line of the LOI and pollen curves represents one sample.



Clonogenic cytotoxicity testing by microdrop encapsulation

Blane Goguen and Nancy Kedersha

Cytotoxicity is defined as the *in situ* measurement of cellular function in response to a toxic substance. Cytotoxicity assays are widely used in drug screening and development research to minimize the number of compounds on which expensive mammalian animal model toxicology studies are performed.

CYTOTOXICITY assays are commonly based on one of several parameters, such as cellular metabolism, membrane integrity and cell growth. Various aspects of cell metabolism can be measured by 'vital' stains, such as rhodamine 123, acridine orange, calcein or 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF), which require different metabolic activities or uptake¹. Many are dependent on cellular activity rather than viability. Membrane integrity is required by living cells and can be assessed by dye exclusion (Trypan blue, propidium iodide, the Hoechst dyes) or chromium release. However, while toxins may leave these cells unable to grow and divide and consequently rendered useless, these assays show cell 'viability', giving an inaccurate measure of drug efficacy. Therefore, measurement of cell growth is a more accurate means of determining long-term toxicity of a substance. Growth, as defined by DNA replication, can be measured through the incorporation of tritiated thymidine. Such assays are suitable in the short term, but are inappropriate for determining the toxicity of microtubule-disrupting agents or DNA alkylating agents, which may require more than one division before the toxic effects are apparent.

The best assays to determine long-term cytotoxic effects are those that are clonogenic, measuring colony growth from a single cell. One drawback of conventional assays is the growth lag that occurs when cells are introduced into low-density culture, which can lengthen assay time. Moreover, during the lag period cells may be markedly less sensitive to toxins than are actively dividing

cells. Here, we report a clonogenic assay using microdrop technology that eliminates the lag period and is suitable for both adherent and non-adherent cells.

Gel microdrop technology

Gel microdrops themselves provide individual microscopic testing regions where a variety of specific clonal cellular function assays can be performed. Studies show that metabolism, protein secretion and antibiotic susceptibility can be measured at the single cell level in gel microdrops²⁻⁵. The small size (<100 µm diameter) of the drops allows them to be analysed by flow cytometry.

The porosity of the gel microdrops allows rapid diffusion of molecules into the gel microdrop matrix. In about 5 min, for example, a 100-µm diameter gel microdrop will reach 99 per cent of the external concentration of an antibody (MW 160 kD): smaller molecules equilibrate even more rapidly².

Preparation of gel microdrops

Gel microdrop cell encapsulation can be performed with the company's growth kit, which contains 3.2 per cent agarose (CelGel) and dimethylpolysiloxane oil (CelMix) in aliquots for performing cell encapsulation. The cells and medium are added to the agarose at 37 °C. This mixture is then added dropwise to dimethylpolysiloxane oil and stirred. An emulsion forms almost immediately upon addition.

After this addition is completed, the emulsion is cooled in an ice bath for 10 min to solidify the gel. Once solidified, the oil can be washed from the drops and the process is complete. The microdrops are robust and can be handled like cells. They can, for example, be dispensed to microplate wells containing different toxin concentrations and incubated. Microdrops can also be centrifuged several times without collapsing.

The mean diameter of the drops is determined by the type of agarose selected and the characteristics of the oil (viscosity, clarity and hydrophobicity), combined with a precise rate of stirring. Dis-

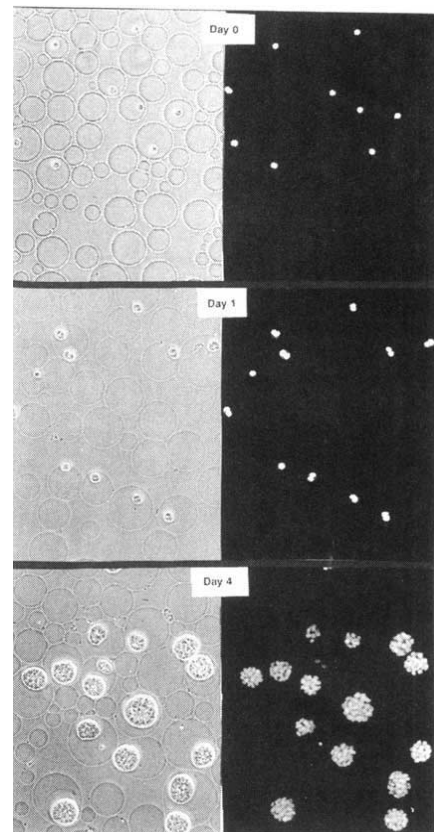


FIG. 2 Growth of cells in gel microdrops. Growth may be monitored directly using phase-contrast and immunofluorescent microscopy. Cells and colonies are readily seen within drops.

persion around the mean is narrow and reproducible as determined by a multisizer particle analyser (Coulter Corporation, Miami, Florida)³. The mean drop diameter can be varied depending on the cell type (prokaryote versus eukaryote) and application. In a growth assay (Fig. 1), drop diameters should be significantly larger than cell diameters to allow room for cell division. The drops will have sufficient volume to contain the cells produced as a result of colony growth.

Poisson statistics adequately describe the frequency-of-occurrence of single cells within the microdrops in the same manner as they do in determining frequency-of-occurrence in microtitre plate and Petri dish inoculation for growth assays. Consequently, to obtain gel microdrops with a high

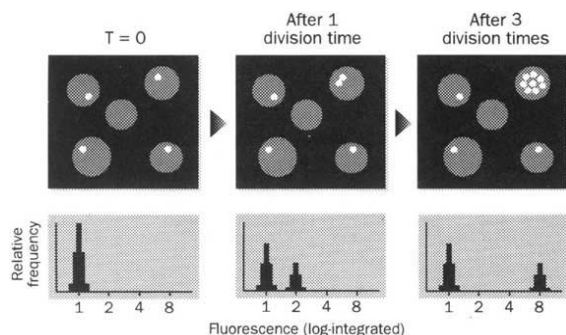


FIG.1 Growth assay.

probability of containing zero or one initial cells, only 10 to 20 per cent of the microdrops would be occupied, in a manner analogous to limited dilution cloning. In this case, approximately 3×10^6 gel microdrops with a mean diameter of 80 μm are formed per millilitre of agarose⁵. Approximately 10^6 cells were added for this assay. With this number of cells, in this volume of agarose, Poisson statistics predict that 90 per cent of the microdrops will contain a single cell; the rest of the occupied microdrops will contain multiple cells.

Analysis of growth

A375 cells (malignant melanoma cells, CRL 1619, American Type Culture Collection, Rockville, Maryland) were encapsulated into micro-drops and their growth into colonies was monitored using light microscopy and flow cytometry. Aliquots were taken daily from day 0 to day 4, fixed and stained with Hoechst 33258, and viewed by phase-contrast and fluorescent microscopy (Fig. 2). Colony growth was apparent using both methods, while the Hoechst-stained nuclei made it easier to count the number of cells in each colony. Note that by day 1 the cells had divided. When using conventional plating methods, these (normally adherent) cells show a growth lag of at least 24 h (ref. 6), which is eliminated in the encapsulated cells.

Growth into colonies was also assessed using flow cytometry. The most sensitive parameters for discriminating between single cells and colonies are side scatter (SSC) and propidium iodide fluorescence. Side scatter is proportional to granularity, which increases along with the increase in cell nuclei and membranes that accompany mitotic growth. Propidium iodide (PI) staining of nuclei acids results in increased fluorescence proportional to DNA synthesis. Displayed together as contour plots (Fig. 3), these two parameters can detect even a single division

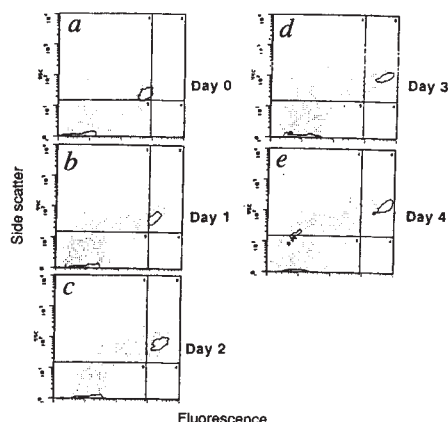


FIG. 3 Analysis of growth by fluorescence activated cell sorting (FACS). Both the side scatter (log scale, x-axis) and fluorescence (log scale, y-axis, propidium iodide (PI) staining) intensities increase as single cells form colonies. The contour plots show a shift upwards and to the right indicating an increase in both side scatter and PI fluorescence as days pass.

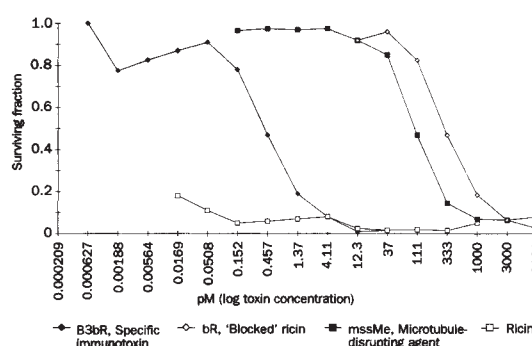


FIG. 4 Encapsulated A375 cells exposed to different toxins and analysed after 4 days of growth: —◆— specific immunotoxin, —■— microtubule-disrupting agent, —□— ricin and —◇— 'blocked' ricin. Data plotted as surviving fraction versus log toxin concentration.

(Fig. 3a and b), which was verified by microscopy (see Fig. 2) on the same specimen. A progressive increase in both SSC and PI fluorescence (Fig. 3c–e) was observed as the number of cells per colony increased. In a cytotoxicity assay, the endpoint can thus be varied according to the nature of the toxin tested. Eight cells per four divisions is often used in clonogenic assays and can readily be accommodated in this assay. The limiting factor is the time at which the cells outgrow the drops (after day 4 in this assay); this varies according to the size and adhesiveness of the particular cell line being tested.

An application of this assay for cytotoxicity testing is shown in Fig. 4. A375 cells were encapsulated, plated in a 24-well plate, and exposed to different toxins over a range of concentrations for 24 h. The toxins were washed off by centrifugation and cells allowed to grow into colonies for 4 days, then fixed and stained and analysed by flow cytometry. An analysis window was determined around the colonies using the zero-growth point and the no-toxin control as standards. From each sample, events falling within the analysis window were scored as colonies. A calculation was made (colony events/total events) at each point to determine the surviving fraction, which was then plotted against toxin concentration (Fig 4). These data were used to determine the IC_{50} of each different toxin. The values obtained were in good agreement with results obtained using a conventional colony-counting assay run in parallel on the same cells (data not shown).

Discussion

Gel microdrop technology provides a new and rapid method of cellular analysis at the single cell level. In this study, we have observed colony growth and its disruption as a measure of cytotoxicity. The assay demonstrated clonal growth without the growth lag that is associated with conventional clonal growth assays. In addition, the use of gel microdrops provides a non-radioactive testing method. A full day was saved using the microdrops due to a 24-h growth lag associated with the A375 cell line.

Gel microdrop technology can increase the speed of toxicity studies, which in turn

may allow new drugs to be brought to market faster and at a reduced cost. The gel microdrop assay could also reduce the need for animal tests that involve, for example, the use of maximum tolerated dose, or MTD bioassays, which are at present under scrutiny by a US National Research Council committee⁷.

In a clinical setting, these savings in time could significantly accelerate the identification and appropriate treatment of drug-resistant microorganisms. Preliminary studies using a clonal growth assay with microdrop-encapsulated tuberculosis cells show promise in providing a rapid means of testing microorganisms for susceptibility to antibiotics.

Clonal growth is just one of the many uses for gel microdrops. Another application is hybridoma selection using immunofluorescence at the single cell level. Secreted antibody from the encapsulated hybridoma cell is captured on immunobeads incorporated in the microdrop and a fluorescent second antibody is added. A sorting flow cytometer can then be used to sort high producing cells from poor producers. □

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Microbiology marketplace

Tools for the microbiologist featured this week include an all-in-one instrument for processing media and autoclaving labware, viral swab transport systems, a portable microplate reader, culture collections and safety videos.

AGARCLAV is the new **all-in-one instrument for media processing and autoclaving** from Integra Biosciences (*Reader Service No. 101*). Up to ten individual programmes can



All in one media processor and autoclave.

be entered and stored. The duration, temperature ($\pm 0.5^\circ\text{C}$) and pressure are controlled, minimizing thermal stress on the medium, and a three-speed magnetic stirring system is built-in, complete with over-temperature compensation. Depending on the model chosen, the company says that up to 10 litres of agar-containing culture medium can be processed in 75 min. A stainless steel cuvette contains the medium and stands in a water bath with integral heating and cooling elements, located outside the chamber walls for simple cleaning. Petri dishes can be filled directly with a dosing pump, or Agarclav can be integrated into the Tecnomatic automatic processing line available from Integra Biosciences. For autoclaving bottles, glassware, instruments and other laboratory equipment, the cuvette is replaced with a perforated stainless steel tray. The steam and air mixture used for aeration and ventilation is exhausted through a waste system to avoid contamination of the laboratory.

A new **fermentor/bioreactor system** has been developed by New Brunswick Scientific for the production of secreted products from microbial, animal or plant cells (*Reader Service No. 102*). Designed to be flexible, the BioFlo 3000 can be converted from a bacterial fermentor to a dedicated cell culture system by selecting the appropriate mode on the touch keypad screen and substituting the interchangeable vessel. For fermentation, a built-in, two-gas control system enriches DO levels by addition of pure oxygen and/or air. For cell culture, an interactive four-gas control system regulates pH and DO. BioFlo

3000 is PC-ready and available with a variety of vessel types and impeller designs, including a packed-basket capable of producing animal cells to 10^8 cells cm^{-3} of basket volume, NBS says.

One of the difficulties with **viral swab transport systems** is their decreasing effectiveness with time. A new design now available from Bibby Sterilin provides swabs with a transport tube which contains dehydrated antibiotics and an isolated reservoir of liquid reagent (*Reader Service No. 103*). With this set-up, the company says that swabs can be stored for up to 24 months before use. The new design is available for chlamydia and virus transport systems. The final medium is prepared only at the time of collection of the specimen. The liquid medium in a squeeze bulb at the top of the transport tube is released to mix with the dehydrated antibiotics at the base of the tube. For viral specimens, a medium containing buffered tryptose phosphate broth, gelatin, chloramphenicol and cyclohexamide is provided. For chlamydial specimens, tubes are available with a medium formula of sucrose, sodium phosphate, potassium phosphate, glutamic acid, gentamycin and amphotericin. A foam pad at the bottom of the transport tube absorbs the medium, and the swab is then placed in the tube. When the sample reaches the laboratory, solution or growth medium is added to the tube, which is vortexed to release infective material.

■ Means of measurement

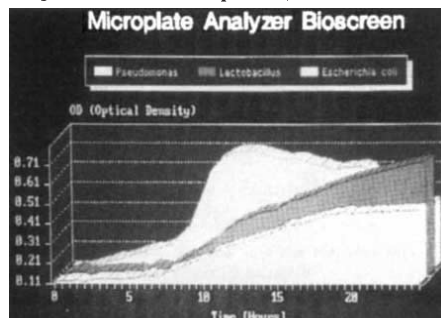
BioResearch Ireland has launched the PortaReader, a low-cost, **portable microplate reader** that is intended for use with enzyme immunoassays (diagnostic) or other colorimetric assays that require measurement at wavelengths of 450 nm or 490 nm (*Reader Service No. 104*). For portability and storage, the PortaReader is conveniently



PortaReader — suitable for laboratory or field-test operations.

designed inside a carrying case and can be powered by battery or mains. As such, it is suitable for laboratory or field operations. Manual reading is designed to be simple and, if necessary, the PortaReader can be interfaced to external devices such as a printer or personal computer.

Bioscreen is the new **robot analyzer** from Labsystems that is designed for microbiologists and which is capable of performing up to 200 microbiological incubation tests simultaneously by using disposable multiwell plates (*Reader Service*



Robot analyzer for multiwell plates.

No. 105). In addition, Bioscreen software converts kinetically measured optical density data to the required report format, including growth curves, concentrations and bacterial counts. It is also possible to use other commercially available software packages such as Lotus and Excel for additional data handling, if required. As an open system, Bioscreen can handle a variety of test layouts and can be used with various microorganisms or liquid media. Bioscreen enables automated diluting, dispensing, incubating, shaking and kinetic O.D. measurements in 200 wells. The incubation temperature is programmable over the 15–55 $^\circ\text{C}$ range and for up to 300 h.

■ Catalogues and videos

The American Type Culture Collection has released the latest edition of the ATCC *Catalogue of Plant Viruses and Antisera* (*Reader Service No. 106*). The 84-page **catalogue** provides information on virus and viroid strains, polyclonal antisera and molecularly cloned viruses available from ATCC. Printed copies of the catalogue are available free of charge in the USA, and for US\$5.80 to all foreign locations. The catalogue is also available on diskette, CD-ROM and via online access.

Over 18,000 of the 40,000 **strains and plasmids** available in the Belgian Coordinated Collections of Microorganisms (BCCM) are listed in four new catalogues

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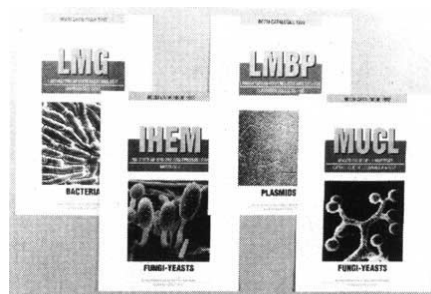
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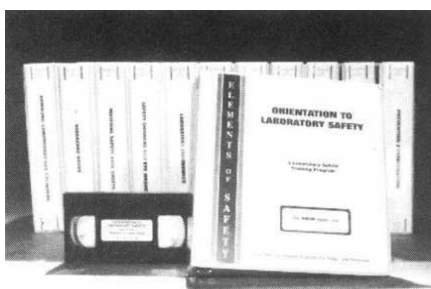
Reader Service No.23



BCCM's collection of bacteria, yeasts fungi and plasmids.

(Reader Service No. 107). Data on applications, growth conditions, pathogenicity of over 7,000 bacteria of biotechnological, agricultural or medical importance (LGM) and 11,000 filamentous and yeast fungi of biomedical (IHEM) and agricultural or industrial (MUCL) importance are described. A selection of 330 plasmids (LMBP), which can replicate in *Escherichia coli*, mainly constructed for cloning and high-level expression of coding sequences in bacteria, yeasts, fungi and animal cells are described in full molecular detail. Functional features are displayed on a circular map, in colour. A brochure illustrating the broad spectrum of microbiological services of the BCCM such as identification, molecular fingerprinting, safe deposit, patent deposit and contract research is also available.

Twelve videotapes each addressing a specific area of laboratory safety make up the the new **safety training programme** that is available from Lux Scientific (Reader Service No. 108). The 8-12-minute videos



Lux Scientific — putting safety first.

cover a range of topics, including *Orientation to Laboratory Safety*, *Laboratory Ergonomics*, *Planning for Laboratory Emergencies*, *Preventing Contamination* and eight other areas of laboratory safety techniques. It is hoped that the presentations will assist laboratories in meeting new OSHA safety regulations, as well as helping to provide a safer working environment. Tapes can be purchased individually or as a complete package.

■ Cytotoxicity testing

Amersham Life Science has introduced complete **assay kits for measuring cytochrome P450 induction**, which are based on nonradioactive western blotting

using enhanced chemiluminescence (Reader Service No. 109). The ECL cytochrome P450 assay range is designed to be comprehensive, covering five P450 isoenzymes in addition to P450 reductase. Hard-copy results recorded on film can be obtained within two days.

Packard has introduced a new procedure to measure both ^3H thymidine cell proliferation and ^{51}Cr release cytotoxicity on a single instrument (Reader Service No. 110). Traditionally, these assays have required the use of two counters — a liquid scintillation counter for ^3H and a gamma counter for ^{51}Cr counting. The introduction of the SpotWell-96 spotting plate allows the use of one instrument, the Matrix, for both applications. Made of stainless steel, the plate has a standard 8×12 microplate pattern and is surrounded by a hydrophobic paint that keeps the sample within the spotting area. Up to 50 µl of cell culture media from a cytotoxicity experiment can be pipetted into each well. After drying, the samples are counted in the Matrix (which counts 96 samples simultaneously) without scintillation cocktail. These same benefits can be applied to counting ^3H thymidine uptake.

■ Detection aids

Kirkegaard & Perry Laboratories has introduced a new affinity purified **antibody to *Escherichia coli* that is specific to *E. coli* serotype 0157:H7** — a food-borne pathogen that causes haemorrhagic colitis and haemolytic uraemic syndrome (Reader Service No. 111). The company says that affinity purification from goat antiserum enhances specificity and minimizes cross-reactivity. The antibody recognizes 25 strains of *E. coli* 0157:H7 and is supplied unlabelled for use as a capture antibody, labelled with peroxidase or phosphatase for microwell ELISAs, western blotting and immunohistology, or labelled with fluorescein for microscopic analysis.

The new GEN-ETI-K tests from Incstar include kits for the **detection of cytomegalovirus, human immunodeficiency virus, hepatitis D (delta virus) and cystic fibrosis**, in addition to a generic DNA detection kit (Reader Service No. 112). The kits use an enzyme immunoassay format as a method for detecting amplified DNA formed by the hybridization of target DNA with specific probes. They utilize a specific monoclonal antibody to detect the double-stranded DNA. Based on a convenient microplate format, the assays detect the hybridization event in less than 4 h, independent of the nucleic acid sequence involved, Incstar says. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal.